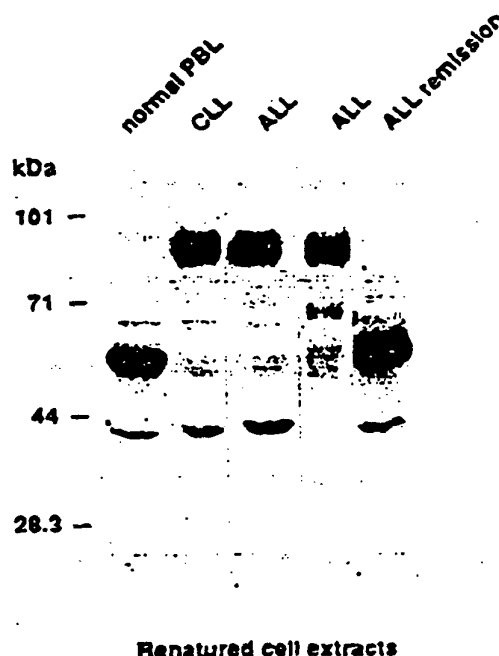**PCT**

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(57) Abstract

New nuclear kinases that are regulated by signal transduction and that participate in phosphorylation cascades which regulate transcription and related methods for regulating transcription. The novel nuclear kinases play a vital role in gene expression, particularly with regard to leukemia in humans. The kinase is (i) substantially exclusively intranuclearly localized; (ii) capable of autophosphorylation; (iii) selectively bindable with antibodies raised against the RING3 portion of GST-RING3; (iv) of a molecular weight of from about 82.5 to about 92.7 kilodaltons; and (v) includes peptide sequences Asp-Ser-Asn Pro-Asp-Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-Pro-Thr-Thr-Leu and Ala-Val-His-Glu-Gln-Leu-Ala-Ala-Leu-Ser-Gln-Ala-Pro. The invention features a method of detecting leukemic cells in a biological sample, the method includes measuring the activity of a RING3-related kinase in the sample, an increase in the activity relative to a control being indicative of the presence of leukemic cells.

Renatured cell extracts

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NUCLEAR LOCALIZED TRANSCRIPTION FACTOR KINASE AND DIAGNOSTIC ASSAYS RELATED THERETO

Background of the Invention

5   The study of how the phosphorylation of proteins
by protein kinases regulates eukaryotic transcription has
enjoyed rapid progress. It has long been known that many
extracellular receptors transfer structural information
from hormones and growth factors into phosphorylation
10 information. A well understood and growing set of
cytosolic kinases, including protein kinase A, protein
kinase C, calcium calmodulin-dependent kinases, oncogene-
encoded kinases, casein kinase II, glycogen synthase
kinase III and the erk-encoded family of kinases, have
15 been shown to contain biochemical signals destined for
the nucleus (Czech et al., 1988; Bohmann, 1990; Blenis,
1991; Jackson, 1992). In the nucleus, phosphorylation
has been shown to affect both the DNA-binding activity
and the transcriptional activation of several
20 transcription factors, some positively and some
negatively (Hunter and Karin, 1992). Activated
transcription of "immediate early genes" seems to be
conducted primarily through phosphorylation.

        However, efforts to date to delineate pathways of
25 information flow from receptor to promoter have tended to
focus on the physical extension of cytosolic signalling
cascades into the nucleus, for example, by demonstrations
of nucleus-cytosol partitioning of transcription factor
substrates and nuclear forms of cytosolic kinases (Hunter
30 and Karin, 1992). Cytosolic protein kinase C has been
shown to phosphorylate the NFkB-IkB complex in the
cytosol, leading to dissociation of the complex,
whereupon transcriptionally active NFkB partitions into
the nucleus (Baeuerle and Baltimore, 1988a; b).
35 Furthermore, a number of kinases that are activated by

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extracellular signals appear to translocate to the
nuclear membrane (or to a nuclear fraction) upon
mitogenic stimulation of the cell, notably protein kinase
A (Nigg et al., 1985; Adams et al., 1991), protein kinase
5   C (Leach et al., 1989), microtubule-associated
protein/myelin basic protein (MAP/MBP) kinase and
ribosomal S6 kinase (RSK) (Chen et al., 1992). Some
kinases that phosphorylate nuclear targets seem to be
activated in the cytosol, such as the cellular homolog of
10 c-abl (Van Etten et al., 1989; Dikstein et al., 1992;
Kipreos and Wang, 1992); whereas the partitioning
behavior of others is either not known to be regulated,
such as FER (Hao et al., 1991) and Sp1 kinase (Jackson et
al., 1990); or is poorly understood, such as the
15 transcription complex-associated kinase that
phosphorylates the C-terminal domain of RNA polymerase II
(Feaver et al., 1991; Lue et al., 1992) and $p33^{cdk2}$, which
is present in a complex with the transcription factor
E2F, cyclin A and p107 (Devoto et al., 1992).
20   While these physical translocations are
undoubtedly important features of signal transduction,
they provide no suggestion that there might also exist
signalling kinases that are downstream targets of
cytosolic kinases but that are restricted solely to the
25 nucleus.

                    Summary of the Invention
        Nuclear kinases that are regulated by signal
transduction and that participate in phosphorylation
cascades which regulate transcription and related methods
30 for regulating transcription and detecting disease states
are described. The novel nuclear kinases play a vital
role in gene expression, particularly with regard to the
expression of genes involved in leukemia in humans.

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Described herein is a novel kinase characterized
as follows: (i) substantially exclusively intranuclearly
localized; (ii) capable of autophosphorylation; (iii)
selectively bindable with antibodies raised against the
5 RING3 portion of GST-RING3; (iv) of a molecular weight of
from about 82.5 to about 92.7 kilodaltons; and (v)
includes peptide sequences Asp-Ser-Asn-Pro-Asp-Glu-Ile-
Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-Pro-Thr-Thr-Leu (SEQ ID
NO: 1) and Ala-Val-His-Glu-Gln-Leu-Ala-Ala-Leu-Ser-Gln-
10 Ala-Pro (SEQ ID NO: 2). This kinase is referred to
herein as the "90 kD kinase." The 90 kD kinase has the
additional characteristic of being able to phosphorylate
a myelin basic protein substrate *in vitro*. The 90 kD
kinase is closely related to human RING3 (herein RING3)
15 described in Beck et al. (1991). The 90 kD kinase may
actually be the protein identified as human RING3. In
any event, the 90 kD kinase is an example of a "RING3-
related kinase." By "RING3-related kinase" is meant the
90 kD kinase described herein, RING3, and Genbank
20 Accession D26362. These proteins are members of a family
of proteins (which may have other members) that can
autophosphorylate, phosphorylate myelin basic protein *in
vitro*, are part of a signalling pathway, and are highly
homologous (at the protein sequence level) to human
25 RING3. Included in the family are allelic variants of
the 90 kD kinase, human RING3, and the protein identified
by Genbank Accession D26362.
    The invention features a biochemical assay for the
presence of leukemic cells. The assay entails measuring
30 the activity of the above-described 90 kD kinase (or
RING3) in a biological sample comprising hematopoietic
cells (e.g., a blood sample, a bone marrow sample, or a
spleen sample). The assay can be used to diagnose acute
lymphocytic leukemia, chronic lymphocytic leukemia, acute
35 myelogenous leukemia, and other forms of leukemia. By

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"leukemic cells" is meant abnormal hematopoietic cells
generally having the following characteristics: (1) poor
responsiveness to normal regulatory mechanisms; (2)
diminished capacity for normal differentiation; (3)
5   ability to expand at the expense of normal lymphoid or
myeloid cell lines; and (4) ability to suppress or
interfere with the growth of normal lymphoid or myeloid
cells. Leukemic cells are cells which overproliferate
relative to normal hematopoietic cells. The method of
10 the invention can be used to detect the presence of
myeloid or lymphoid leukemias. In addition to detecting
leukemic cells, the method of the invention may be used
to provide a measure of the agressiveness of leukemic
cell overproliferation. Thus, both the relative number
15 of leukemic cells and their potential for rapid
proliferation may be estimated.

        The level of 90 kD kinase activity can be used to
judge the stage of a patient's leukemic condition. It
may be used for the initial diagnosis of leukemia or to
20 determine whether (or if) a patient already diagnosed
with leukemia, but in remission, is at risk for relapse.

        There are a wide range of assays which can be used
measure 90 kD kinase activity. The autophosphorylation
activity of the 90 kD kinase is correlated with its
25 activation. Thus, one can measure activated 90 kD kinase
by measuring the level of autophosphorylation. This can
be done by allowing autophosphorylation to occur in the
presence $[\gamma\text{-}^{32}P]ATP$ or some other detectable label.
Activity of the 90 kD kinase can also be determined by
30 measuring the its ability to phosphorylate a substrate
such as myosin light chain kinase peptide, Kemptide, or
myelin basic protein.

        Alternatively, an antibody specific for the
phosphorylated form of 90 kD kinase can be used to
35 measure 90 kD kinase activity by measuring the level of

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phosphorylated 90 kD kinase (or RING3-related kinase)
using an ELISA assay or other antibody based protein
detection and quantification assays.

The 90 kD kinase has some homology to fsh
5   (Drosophila) and RING3 (human). Human RING3 was found to
posses protein kinase activity. RING3 is attributed with
a role consistent with that of the novel kinase. In some
cases assays used to detect RING3 or RING3 activity can
be used to detect 90 kD kinase or 90 kD kinase activity.

10   Thus, the invention features a method of detecting
leukemic cells in a biological sample. The method
includes measuring the activity of a RING3-related kinase
in the sample, an increase in the activity relative to
that present in a control being indicative of the
15 presence of leukemic cells.

Suitable controls include similar biological
samples known to contain little or no active 90 kD
kinase, e.g., a biological sample from a patient known
not to be suffering from leukemia (or other cancers).
20 Preferably the biological sample is of the same type as
the test sample (e.g., both are whole blood samples).
Other suitable controls are non-biological standards
known not to contain significant levels of active 90 kD
kinase. Test kits which include such a negative control
25 sample and/or a postive control sample (a sample known to
contian active 90 kD kinase (or RING3) are an aspect of
the present invention.

In various preferred embodiments the biological
sample comprises lymphocytes, the activity is measured by
30 assaying the autophosphorylation activity of a RING3-
related kinase, the activity is measured by assaying
phosphorylation of a substrate of a RING3-related kinase,
the activity is measured by measuring autophosphorylation
activity, and autophosphorylation activity is measured by
35 immunological methods.

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In another preferred embodiment, the RING3-related kinase activity is measured in a first and a second biological sample, a higher level of RING3-related kinase activity in the first biological sample relative to the
5 second biological sample indicating the presence of more leukemic cells in the first biological sample than in the second biological sample. In even more preferred embodiments the first and second biological samples are obtained from the same patient at different times, and
10 the second biological sample is obtained from an individual that is not suffering from a leukemic condition.

In another aspect, the invention features a kit for detecting leukemic cells in a biological sample, the
15 kit includes a substantially pure antibody that specifically recognizes an autophosphorylated form of a RING3-related kinase.

Other features and advantages of the invention will be apparent from the following detailed description
20 thereof, and from the claims.

Brief Description of the Drawings

Fig. 1A: Autophosphorylation of a 90 kD kinase in Jurkat cells is increased by forskolin treatment. Nuclear extracts from forskolin-treated and control
25 Jurkat cells were fractionated on phosphocellulose columns. The columns were eluted stepwise with 0.3 M and 0.6 M sodium chloride in buffer. Eluted proteins were precipitated with trichloroacetic acid, solubilized in sodium dodecyl sulfate, separated by gel electrophoresis,
30 electroblotted to nitrocellulose, denatured in guanidine hydrochloride, renatured in buffer, and assayed by autophosphorylation in the presence of $[\gamma-^{32}P]ATP$ and magnesium. Autoradiographs of blots are shown. Unfractionated nuclear extracts (UF) are included for
35 comparison.

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Fig. 1B: Time course of increased
autophosphorylation that is induced by forskolin.   Jurkat
cells were stimulated with 50 μM forskolin for 0, 3, 6,
9, 12, 15, and 30 minutes.   Nuclear extracts of these
5   cells were applied to phosphocellulose.   Proteins were
eluted with 0.3 M sodium chloride and assayed as
described for Fig. 1A.

Fig. 1C: Treatments that affect 90 kD
autophosphorylation in A431 cells.   A431 cells were
10   treated with epidermal growth factor (1 nM), calf serum
(20%), forskolin (25 μM) or heat (42°C) for 30 minutes.
Nuclear extracts of treated cells and control cells were
applied to phosphocellulose.   Proteins were eluted with 1
M sodium chloride and assayed as described for Fig. 1A.
15   The 90 kD kinase is identified with an arrow.

Fig. 2:   The 90 kD kinase activity is localized to
the nucleus in HeLa cells.   A homogenate of HeLa cells
was prepared in the presence of phosphatase inhibitors
and centrifuged at 100,00 x g for one hour at 4°C.   Equal
20   amounts of protein (50 μg) from this supernatant (the
cell cytosol) and a HeLa nuclear extract were separated
by gel electrophoresis and assayed as described for Fig.
1A.   The 90 kD kinase is identified with an arrow.

Fig. 3: The 90 kD kinase activity HeLa cells has
25   an apparent molecular weight of 350,000 by size
exclusion chromatography.   Proteins in HeLa nuclear
extract were separated by size exclusion chromatography
at physiological ionic strength and assayed as described
for Fig. 1A.   The void volume of the column and molecular
30   weight standards (for size exclusion chromatography) for
immunoglobulin (158,000) and ovalbumin (44,000) are
indicated above the relevant fractions, and molecular
weight standards (for gel electrophoresis) are indicated
to the left of the figure.   The 90 kD kinase is
35   identified with an arrow.

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Fig. 4: Renatured 90 kD kinase was tested for its
ability to phosphorylate myelin basic protein. Strips of
nitrocellulose upon which the renatured kinase was
immobilized were incubated in Eppendorf tubes with a

5 solution of myelin basic protein, radioactive ATP and
magnesium in buffer. After one hour at 30°C, proteins
were solubilized at 100°C in sodium dodecyl sulfate,
separated by gel electrophoresis and visualized by
Coomassie blue stain. An autoradiograph of

10 phosphorylated myelin basic protein is shown in the
figure. Three species of kinase were tested in
triplicate: the activity in unfractionated HeLa nuclear
extract (UF), the activity in a 0.3 M sodium chloride
fraction from phosphocellulose-fractionated HeLa nuclear

15 extract, and the activity in a 1.0 M sodium chloride
fraction of the same extract.

Fig. 5A: Fractionation of autophosphorylating 90
kD kinase by phosphocellulose (P-11) chromatography.
HeLa nuclear extract was applied to phosphocellulose and

20 eluted stepwise in 0.1 M increments with 0.2 M to 0.8 M
sodium chloride in buffer. Eluted proteins were assayed
as described for Fig. 1A. Unfractionated nuclear extract
is included for comparison at the left of the figure.
Molecular weight markers are shown at the extreme left

25 and an arrow indicates the average apparent molecular
mass of the 90 kD autophosphorylating species.

Fig. 5B: Substrate-directed phosphorylation
activity of the 90 kD kinase is associated with the
slower-migrating forms identified by autophosphorylation,

30 not with the faster-migrating forms. The same fractions
from Fig. 5A were renatured and used to phosphorylate
myelin basic protein as described in Fig. 4.

Fig. 6A: Autophosphorylated amino acids are
serine and threonine, not tyrosine. HeLa nuclear extract

35 was applied to phosphocellulose and eluted with 0.3 M

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sodium chloride. The 90 kD kinase was isolated,
renatured as described for Fig. 1A, and
autophosphorylated with $[\gamma-^{32}P]ATP$. The radioactive
kinase was then hydrolyzed in 6N hydrochloric acid and
5   its constituent radioactive amino acids were separated by
electrophoresis on silica. An autoradiograph of the
silica sheet is shown. Authentic phosphoaminoacid
standards were run in the same sample and visualized with
ninhydrin to identify the radioactive spots.

10   Fig. 6B: Myelin basic protein is phosphorylated
only on serine. Left side: Myelin basic protein was
phosphorylated with renatured 90 kD kinase as described
for Fig. 4, the hydrolyzed and separated as described for
Fig. 6A. Right side: As a control for tyrosine
15   phosphorylation, myelin basic protein was also
phosphorylated in a separate reaction with recombinant
$p43^{v-abl}$ protein.

             Fig. 7: Immune sera to fsh and RING3
immunoprecipitate the 90 kD autophosphorylation activity.
20   The cDNAs for fsh and RING3 were obtained and fusions
with glutathione-S-transferase (GST) were constructed.
These constructs were expressed in *E. coli*, the fusion
proteins were purified and used to immunize rabbits. The
90 kD kinase was immunoprecipitated from HeLa nuclear
25   extracts with these antisera and assayed by
autophosphorylation as described for Fig. 1A. Pre-immune
sera were not capable of immunoprecipitating this
activity. Autoradiographs of 90 kD kinase renatured from
immunoprecipitates of preimmune and immune sera against
30   RING3 (above) and fsh (below) are shown.

             Fig. 8: Immunofluorescence with antibodies
against RING3 confirm nuclear localization of 90 kD
kinase in HeLa cells. HeLa cells were grown on
coverslips and fixed by standard methods. Coverslips
35   were incubated with preimmune serum or with affinity-

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purified antibodies against GST fusion proteins of RING3
or fsh. Goat anti-rabbit secondary antibodies conjugated
to fluorescein were used to visualize the primary
antibodies.

5   Fig. 9A: Autophosphorylation activity of the 90 kD
kinase is increased in patients suffering from leukemia,
but not in normal patients or leukemia patients in
remission. The position of molecular weight markers is
shown at the left. The position of the 90 kD kinase is
10 indicated by the arrow.

        Fig. 9B: Anti-RING3 antibody immunoprecipitates 90
kD kinase autophosphorylation activity from leukemic
patients. The position of molecular weight markers is
shown at the left. The position of the 90 kD kinase is
15 indicated by the arrow.

                    Detailed Description

        Regulated transcription of eukaryotic nuclear
genes depends in part on the accurate processing of
information about the extracellular milieu, especially
20 hormone, growth factor, drug, or other ligand binding to
plasma membrane (cell surface) receptors. This
information is processed through multiple kinase cascades
within the cell, ultimately giving rise to the activation
of transcription factors in the nucleus. It is of vital
25 interest to identify the kinases that play a role in this
transduction of signals and to delineate the signalling
networks involved.

        In accordance with the invention it was reasoned
that there must be many uncharacterized kinases that are
30 mostly or entirely localized to the nucleus, that receive
signals transduced from the cytosol and then go on to
phosphorylate and activate nuclear components of the
transcription apparatus.

        In the process of arriving at the invention,
35 nuclear extracts of cultured cell lines were screened for

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previously uninvestigated kinases that exhibit regulated
activity and that might phosphorylate transcription
factors. In order to increase the likelihood that such a
kinase is important in transcription, its principal
5   features were considered to include primarily (although
not necessarily exclusively) nuclear localization, and
responsiveness to drugs, hormones or conditions that are
known independently to promote transcription in cultured
cells.

10   A straightforward screen of nuclear extracts of
Jurkat cells, performed by adding magnesium and $[\gamma-^{32}P]ATP$
to the extract, incubating it, and identifying the
proteins by denaturing gel electrophoresis and
autoradiography, did not reveal any obvious differences
15   in phosphorylation between the proteins from cells
stimulated with phorbol myristate
acetate/phytohemagglutinin and controls. Too many
phosphorylated proteins obscured potential differences.

          In accordance with the invention, this problem was
20   addressed and overcome by nitrocellulose electroblotting
gels of electrophoretically separated proteins,
denaturing the blotted proteins in guanidine
hydrochloride (e.g., 7M), renaturing them in buffer and
then probing the blot with
25   $[\gamma-^{32}P]ATP$ (Ferrell and Martin, 1989). This assay
visualized only those kinases which could renature after
gel electrophoresis in sodium dodecyl sulfate and could
autophosphorylate.

          A previously uncharacterized autophosphorylation
30   activity has now been identified in HeLa nuclear extract.
Denaturing polyacrylamide gels of nuclear proteins were
electroblotted to nitrocellulose, renatured and assayed
for autophosphorylated bands by overlaying the blots with
$[\gamma-^{32}P]ATP$ and magnesium ion. Autoradiography of the
35   blots visualized a limited set of kinases, one of which

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was predominantly nuclear and which exhibited rapidly
increased autophosphorylation in forskolin-stimulated
Jurkat cells and both serum and forskolin-stimulated A431
cells.

5   A forskolin analog with greater water solubility
and activity approximately equal for forskolin was used
in these studies. This analog is called 7β-deacetyl-
7β(γ-N-methylpiperazino)-butyryl forskolin and is
supplied by Calbiochem as product number 344273. Because
10 forskolin is a potent activator of adenylate cyclase,
other pharmacological agonists of adenylate cyclase, such
as other derivatives of forskolin, are expected to
stimulate the activity of the 90 kD kinase in a similar
manner. By the same token, other drugs, hormones or
15 growth factors that increase the intracellular activity
of cyclic AMP-dependent protein kinase, which is the
target of the cyclic AMP produced by adenylate cyclase,
are also expected to stimulate the 90 kD kinase. These
other drugs or hormones include membrane-permeable
20 derivatives of cyclic AMP, such as its dibutyryl ester or
"caged" cyclic AMP; isoproterenol, glucagon, epinephrine,
bombesin or other agents that mobilize cyclic AMP in
their responsive cell types; and other as yet
uncharacterized factors in serum that mobilize cyclic
25 AMP. In addition, agents that inhibit phosphodiesterase,
which destroys the cyclic AMP, will increase
intracellular cyclic AMP concentrations and by mobilizing
cyclic AMP-dependent protein kinase, are expected to
increase the activity of the 90 kD kinase.
30 Phosphodiesterase inhibitors include such agents as 3-
isobutyl-1-methylxanthine. Finally, DNA expression
vectors that encode the catalytic subunit of protein
kinase A will also increase the intracellular activity of
protein kinase A and are thereby expected to increase the
35 activity of the 90 kD kinase.

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The kinase activity resolved as a multiplet of
average apparent molecular mass 90 kD on SDS gels.
Phosphocellulose chromatography fractionated the
multiplet into forms of slightly slower and faster
5   mobility. The forms of slightly slower mobility on SDS
gels bound more weakly to phosphocellulose than the forms
of slightly faster mobility, probably due to
hyperphosphorylation, and these exhibited both increased
autophosphorylation and increased substrate-directed
10 phosphorylation if they were isolated from cells that had
been stimulated with serum or forskolin. Analogous
phenomena have been observed with pp70^{S6K} (Price et al.,
1992) and pp90^{rsk} (Vik et al., 1990) S6 kinases, for which
the higher phosphorylated forms exhibit both reduced
15 mobility on SDS gels and increased substrate-directed
phosphorylation. This pattern, in which increased
phosphorylation of the kinase reflects increased
substrate-directed activity, is observed also with
protein kinase C (Mitchell, et al., 1989) and appears to
20 define a subclass of intracellular signalling kinases.

This 90 kD autophosphorylation activity appears to
be ubiquitous, as it was observed in every mammalian cell
line assayed. Its presence in yeast, Drosophila or
plants has not yet been determined. The occurrence of
25 well-resolved autophosphorylating kinases of the same
apparent mobility in a wide variety of cells was
consistent with the fact that the renaturation assay that
was used selected for independently refolding catalytic
domains that do not require protein disulfide isomerase
30 or chaperonin activity to regain function. Such stably
folded kinases are likely to share certain structural
features of folding pathways that are conserved in
different organisms.

Autophosphorylation was dramatically reduced to a
35 basal level when the extracts were prepared in the

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absence of phosphatase inhibitors or when extracts were
warmed to 37°C in the presence of 5 mM magnesium ion,
thereby eliminating differences between extracts prepared
from control or stimulated cells, a frequently observed
5 feature of signal-transducing kinases (Kozma et al.,
1990). Without being bound by any particular theory,
these observations point to the interpretation that the
90 kD kinase is probably rapidly and reversibly
phosphorylated and activated by an intracellular signal
10 transduction pathway. These changes to
autophosphorylation activity were remarkably stable, a
phenomenon that has been observed among certain kinases
involved in signal transduction, such as RSK and MAP
kinases (Ahn et al., 1990).
15   Consistent with a large body of work on "switch
kinases" and nuclear phosphorylation (Ahn and Krebs,
1990; Pelech et al., 1987; 1990) that has failed to
detect phosphotyrosine phosphorylation in the nucleus,
the 90 kD species contained no phosphotyrosine, and
20 phosphorylated its myelin basic protein substrate only on
serine. Myelin basic protein is used frequently as an *in
vitro* substrate for MAP kinases, and its suitability here
suggested that the 90 kD kinase might share some
structural or functional similarities with the members of
25 the MAP kinase family or with the RSK family, which is
immunologically related to the MAP kinases. However, the
90 kD kinase did not phosphorylate a range of substrates,
including peptide substrates for S6 kinase, MAP kinase,
casein kinase II, glycogen synthase kinase III, protein
30 kinase C, calcium-calmodulin-dependent protein kinase, or
Raytide (a general substrate for tyrosine kinases). It
did phosphorylate peptide substrates for smooth muscle
myosin light chain kinase and for protein kinase A and
was active in the absence of cofactors. These substrate
35 peptides have at least two hydrophobic, basic amino acids

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that are positioned two residues toward the N-terminus
from a phosphorylatable serine (Kemp and Pearson, 1990).
Amino acid sequence analysis of the phosphorylation site
and site-directed mutagenesis will provide more
5 information about the substrate requirements of the
kinase. The nuclear localization of the enzyme and its
lack of immunoreactivity with rabbit polyclonal
antibodies to protein kinase C, RSK or MAP kinases
indicated that the 90 kD kinase was unlike other
10 previously characterized kinases.

This question of its identity was most effectively
resolved by purification and microsequencing of the
protein. The initial strategic choice to look for
activities that were renaturable on nitrocellulose after
15 electrophoresis in SDS and electroblotting proved to have
an added benefit of disqualifying those kinases that
rapidly lose activity during purification. The great
stability of the activity made purification easier. THis
characteristic of the 90 kD kinase can be exploited to
20 rapidly carry out partial purifications where required to
accurately measure the level of activated 90 kD kinsase.
Classical protein purification techniques yielded
activity in sufficient purity and quantity to obtain a
microsequence of two internal peptides. One of these
25 peptides produced a significant match with both the
Drosophila fsh gene (DROFSHB) (Haynes et al., 1989) and
with the human RING3 gene (HUMRING3) (Beck et al., 1991),
whereas the other matched only RING3. This result
implied that these genes encoded functional kinases,
30 which has not been reported in the literature.
Bacterially expressed cDNAs for these genes failed to
exhibit autophosphorylation *in vitro* unless they were
incubated with HeLa nuclear extract. Rabbit polyclonal
antibodies raised against two bacterially expressed N-
35 terminal fusions of the human and Drosophila cDNAs with

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glutathione-S-transferase were capable of
immunoprecipitating the original 90 kD
autophosphorylation activity from HeLa nuclear extract,
whereas preimmune sera were not. Open reading frames for
5 fsh identify gene products of molecular weight 110,000
and 205,000 for 5.9 kb and 7.6 kb mRNA transcripts,
respectively (Haynes, et al., 1989), which is at variance
with the apparent molecular mass of the activity isolated
from HeLa nuclear extract. Post-translational
10 proteolytic cleavage of the fsh gene products to give
active kinase may account for this discrepancy; such
proteolytic activation of kinases is well exemplified by
protein kinase C cleavage to yield constitutively active
protein kinase M (Schaap, et al., 1990). The open
15 reading frame for RING3, on the other hand, identifies a
gene product of molecular weight 83,000 (Beck et al.,
1991) which could account for the apparent molecular
masses observed, especially for a phosphorylated gene
product (Kozma et al., 1990).
20   Consistent with the apparently nuclear
localization of the HeLa 90 kD kinase, the RING3 gene
possesses a putative nuclear localization sequence (KKKRK
(SEQ ID NO: 3), at amino acid 508; Beck et al., 1991);
however, this sequence is absent from fsh and lies
25 upstream from a C-terminal domain of homology (170 amino
acids, 72% identity) that the two genes share (Beck et
al., 1991). This region of homology contains some
elements of a putative kinase catalytic domain.

Subdomain Structure

30   The molecular organization of fsh and RING3 is
somewhat unusual. A putative phosphate binding motif
GXXXXGK (SEQ ID NO: 4) (Moller and Amons, 1985) is
located at amino acid residue 809 in fsh, and a putative
ATP-binding motif GXGXXG (SEQ ID NO: 5) (Hanks et al.,
35 1988) is found at amino acid position 880 in fsh and 557

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in RING3. Fifteen residues downstream from the last
glycine of GXGXXG (SEQ ID NO: 5) in fsh and 11 downstream
in RING3 lies a putative catalytic lysine residue, which
is in both cases two residues downstream from an alanine
5 that is conserved in many kinases. This sequence in fsh
(GAG SVG GVG GAG AAG GGN ASK (SEQ ID NO: 6)), is most
highly related to the corresponding subdomains of c-mos
(GAG GFG SVY KAT YRG VPV AIK (SEQ ID NO: 7); Watson et
al., 1982). A run of glutamates 43 residues downstream
10 from the putative catalytic lysine in fsh and 13
downstream in RING3 identifies a putative subdomain III
within a C-terminal region of significant sequence
homology between fsh and RING3. Another motif, DFE, at
position 997 in fsh and 640 in RING3, suggests subdomain
15 VII (DFG). An interesting extended run of serines at the
C-terminus of the open reading frame may function as an
autophosphorylation or autoinhibitory domain (Banerjee et
al., 1990). Despite these sequence similarities, some of
the conserved subdomains that ordinarily occur downstream
20 from the putative DFG motif are missing in fsh and RING3
because the open reading frame ends.

However, lack of certain consensus motifs does not
mean that a particular open reading frame cannot encode a
kinase (Wu et al., 1990; Maru and Witte, 1991).
25 Strangely, in fact, putative subdomains occur in an
appropriate order in the middle of the open reading frame
of each gene, in a different region of significant
homology between fsh and RING3. Here, a motif
YHDIIKXPXXL (SEQ ID NO: 8) suggests subdomain VIB; a
30 motif APEF (SEQ ID NO: 9) in fsh and AQEF (SEQ ID NO: 10)
in RING3 suggests subdomain VIII; a motif DVVAMXRKL (SEQ
ID NO: 11) suggests domain IX; a motif YAKM (SEQ ID NO:
12) in RING3 suggests subdomain X and HRLAEXXXXXXXXHEQLAA
(SEQ ID NO: 13) in RING3 suggests subdomain XI. This
35 unusual overall structure is not incompatible with kinase

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catalytic activity and may have arisen through exon
shuffling. Another signal-transducing kinase, S6 kinase
II from chicken and Xenopus, possesses two catalytic
domains that are homologous to protein kinase C and
5   substrate recognition domains homologous to phosphorylase
kinase. This structure has also been proposed to arise
from exon shuffling (Banerjee et al., 1990). We have
been unable to identify other kinases in the GenBank
database that exhibit similar organization or that have
10 regions of homology with fsh and RING3 that extend much
beyond the individual subdomains discussed here.

Most intriguing is the potential role that these
genes may play in development and transformation. In
Drosophila, mutations in the fsh locus exhibit a homeotic
15 phenotype. The gene is required at two stages in
development: once during early embryogenesis, where there
arises a maternal effect that cannot be rescued by sperm
carrying the wild type allele (Forquignon, 1981), and
again during pupation, where the effect is zygotic and
20 the numbers of progeny with the mutant phenotype
decreases with increasing dose of the wild type allele
(Gans et al., 1980). These two periods are temperature-
sensitive and at the restrictive temperature, the effect
is lethal. At semipermissive temperatures, heterozygous
25 progeny can reach adulthood, but have a high frequency of
organ deficiencies and transformations of the third
thoracic segment to the second thoracic segment. This
phenotype resembles transformations in the bithorax
complex of genes, and indeed, mutations in certain
30 bithorax genes such as trithorax (also called Regulator-
of-bithorax) and Ultrabithorax[130] are closely linked to
fsh and show increased severity with hypodosage of wild
type fsh (Forquignon, 1981). Trithorax (Genbank
accession number M31617), which is required during
35 embryogenesis and later, requires activation by fsh

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(Digan et al., 1986) and has a number of putative zinc
fingers that indicate it is likely to be a transcription
factor (Mazo et al., 1990). In light of this evidence,
we propose that fsh is a kinase that phosphorylates
5   trithorax, thereby activating trithorax directed
transcription of developmentally important genes. The N-
terminal domain of the open reading frame of trithorax
has several phosphorylation motifs of the type
(R/K)(R/K)XX(S/T), which can be recognized by protein
10   kinase A and myosin light chain kinase (Kemp and Pearson,
1990). The substrate specificity of the 90 kD kinase
described above is not inconsistent with an ability to
phosphorylate trithorax.

The biological role of RING3 is less well
15   understood. Its occurrence in the major
histocompatibility complex among a set of recently
characterized genes that do not appear to have a role in
antigen processing suggests that if it is a nuclear
kinase in human cells, there may be a human homolog of
20   trithorax that has zinc fingers and that functions as its
transcription factor substrate. In this regard, a recent
report that maps the breakpoint in chromosomal
translocations of human chromosome 11 has identified an
interrupted gene with significant homology to trithorax
25   (Djabali et al., 1992). This t(4;11)(q21;q23) reciprocal
translocation is associated with acute lymphocytic
leukemia in children, which may represent a biological
consequence of loss-of-function of the human trithorax
homolog. In addition to putative zinc fingers, the
30   homolog has "AT hook" motifs that may be involved in
binding to the minor groove of DNA (Tkachuk et al.,
1992). In-frame fusions of this human homolog, called
the ALL-1 gene (Gu et al., 1992) to the AF-4 gene as a
result of the reciprocal translocation may give rise to a
35   new protein with oncogenic transforming potential

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(Tkachuk et al., 1992; Gu et al., 1992). Alternatively, deletion of important regulatory sites of phosphorylation in ALL-1 by the translocation may account in part for the oncogenic effects. In such a case, we would predict by

5 analogy that alteration-of-function mutations in RING3 might be associated with leukemia as well. Further characterization of the relationship between RING3 and the trithorax homolog might illuminate the genomic organization of RING3 within the major histocompatibility

10 locus and its expression in T-cells (Beck et al., 1991) as well as the emerging parallel relationships between the important players in Drosophila development and human cancer.

Example 1

15 Characterization of Transcription Factor Nuclear Kinases
Materials

Dextran sulfate (average molecular weight 500,000), insulin, glucagon, acidic fibroblast growth factor, reactive green-19 and Cibacron Blue 3GA (Type

20 3000) agarose were from Sigma. Sodium orthovanadate, Tris, trichloroacetic acid, $MgCl_2$ and NaOH were from Fisher. Disodium ATP and NP-40 were from Pharmacia LKB. Guanidine hydrochloride was from Fluka. Phytohemagglutinin-M, phorbol 12-myristate 13-acetate,

25 calcium ionophore A23187, human interleukin-2, (7β-deacetyl-7β-[-N methylpiperazino]-buytyrl)-forskolin dihydrochloride and rat brain protein kinase C were from Calbiochem. Wheat germ extract and rabbit reticulocyte extract systems for in vitro translation were from

30 Promega. Raytide and recombinant $p43^{v-abl}$ were from Oncognene science (Uniondale, NY). In vitro phosphorylation substrates were from Peninsula Laboratories (Belmont, CA). [α- and γ-^{32}P]ATP were from New England Nuclear. Nitrocellulose membranes were from

35 Micron Separations (Westboro, MA) and polyvinyl

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difluoride membranes (PVDF) were from Millipore.
Recombinant protein A-agarose was from Repligen
(Cambridge, MA). Phosphocellulose resin (P-11) was from
Whatman. Polyacrylamide monomer, bisacrylamide and
5 ammonium persulfate were from National Diagnostics
(Manville, NJ). Cell culture media, calf serum,
monoclonal antibody Mab 1.9 against rat brain protein
kinase C and TEMED were from GIBCO-BRL. Fetal bovine
serum for Jurkat cell culture was from Whittaker
10 Bioproducts (Walkersville, MD). Cell lines (HeLa, ATCC
CCL 2; A431, ATCC CRL 1555; Jurkat, ATCC TIB 152; HUT78,
ATCC TIB 161; Mvl Leu, ATCC CCL64; CHO DUKX B1, ATCC CRL
901 0; CV-1, ATCC CCL70; and COS-7, ATCC CRL 1651) were
obtained from ATCC and cultured as advised. Jurkat clone
15 E6-1 (Weiss et al., 1984) and HUT78 (Gazdar et al., 1980)
were obtained through the AIDS Research and Reference
Reagent Program, Division of AIDS, NIAID, NIH. 3T3-L1
fibroblasts (ATCC CL 173) were the kind gift of Michael
P. Czech, University of Massachusetts Medical Center, and
20 were differentiated according to Rubin et al. (1978). An
Epstein-Barr virus-transformed human B-cell line (32D
clone 13) (FitzGerald et al., 1991) was obtained from
Joel Greenberger, University of Massachusetts Medical
Center. Human peripheral blood lymphocytes from normal,
25 healthy volunteers, were the kind gift of John Sullivan
University of Massachusetts Medical Center. Rabbit
polyclonal antisera to purified GST fusion proteins were
prepared by Berkeley Antibody Company (Richmond, CA).
Platelet-derived growth factor was the kind gift of Roger
30 J. Davis, University of Massachusetts Medical Center.
Rabbit anti-rat MAP kinase (erk-1 C-terminal 333-367
amino acids) polyclonal antibody was from Upstate
Biotechnology (Lake Placid, NY) and anti-RSK rabbit
antibody was the kind gift of John Blenis, Harvard
35 Medical School. HeLa nuclear extract was prepared with 1

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mM sodium vanadate and 50mM ß-glycerophosphate after the method of Dignam et al. (1983). All other reagents were from Sigma.

Renaturation assay

5   Proteins were separated in polyacrylamide gels according to Laemmli (1970) and electroblotted to nitrocellulose or PVDF membranes. Blotting was performed in 25 mM Tris, 192 mM glycine, 10% methanol at 100 mA for one hour at room temperature. The blots were incubated

10 in 7 M guanidine HCl, 50mM Tris-HCl pH 8.5, 50 mM DTT and 2 mM EDTA for one hour at room temperature to denature blotted proteins, then transferred to ice-cold buffer containing 100 mM NaCl, 50mM Tris-HCl, 2mM DTT, 2mM EDTA and 0.1% NP-40. Proteins were renatured overnight at 4°C

15 in this buffer. The blots were then blocked with 5% dextran sulfate in the same buffer for one hour at room temperature; then incubated for one hour at room temperature with 0.15 mCi/ml [γ-^{32}P]ATP (25 nM) in 30 mM Tris-HCl pH 8.0, 30 mM MgCl$_2$, 2 mM DTT, 0.2 mM EDTA and

20 0.1% NP-40; and washed sequentially for ten minutes each with: 50 mM Tris-HCl pH 8.0; 0.1% NP-40 in 50 mM Tris-HCl pH 8.0; 50 mM Tris-HCl pH 8.0; 1 M NaOH; 10% acetic acid and then water, after Ferrell and Martin (1989). The blots were then autoradiographed at -80°C.

25   For phosphorylation of protein or peptide substrates in solution, nitrocellulose strips of immobilized, renatured kinase were excised and placed in Eppendorf tubes with 30 mM Hepes pH 8.0, 30 mM MgCl$_2$ (or 1 mM MnCl$_2$), 2 mM DTT, 0.5 mM EDTA, 0.1% NP-40, 10 μM

30 disodium ATP, 0.15 mCi/ml [γ-^{32}P]ATP and 1 mg/ml myelin basic protein or 1 mg/ml peptide substrate in a final volume of 10 μl. Reactions were allowed to proceed for one hour at 30°C, whereupon protein phosphorylation reactions were quenched with SDS sample buffer and heated

35 to 100°C. Phosphorylated protein samples were separated

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by SDS PAGE and visualized by Coomassie Blue staining and
autoradiography. Peptide phosphorylation reactions were
quenched with 4 volumes of ice-cold 10% phosphoric acid.
Phosphorylated peptide samples were applied to 0.1 ml
5 columns of phosphocellulose that had been prequilibrated
with 0.5% phosphoric acid. Columns were washed
extensively with 0.5% phosphoric acid and radioactivity
that remained in the columns was quantified by Cerenkov
counting.

10   Phosphoaminoacid analysis
         HeLa nuclear extract was fractionated on
phosphocellulose and proteins that eluted at 0.3 M and
1.0 M NaCl were precipitated with TCA, washed with
acetone, solubilized in SDS, separated on an 8%
15 polyacrylamide gel, electroblotted to PVDF, denatured in
guanidine Hydrochloride and renatured as described. PVDF
strips were excised, placed in Eppendorf tubes and
incubated with [γ-^{32}P]ATP without unlabelled ATP as
described. The autophosphorylated kinases were then
20 hydrolyzed in 6N HCl at 110°C for one hour, according to
Lewis et al. (1990). The acidic solution was diluted
five-fold with water : methanol (50:50; v:v) and
lyophilized twice. The lyophilization residue was
dissolved in 30% formic acid and spiked with
25 phosphoaminoacid standards. Equal amounts of
radioactivity (about 2000 cpm by Cerenkov counting) were
applied to a cellulose thin layer plate.
Phosphoaminoacids were separated by electrophoresis in
glacial acetic acid: pyridine : water (10:1:89; v:v:v) at
30 pH 3.5 for two hours at 1000 V and visualized with
ninhydrin. Radioactive spots were visualized by
autoradiography.

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Protein purification

HeLa nuclear extract (100 ml) was applied to a
column of reactive green-19 resin (40 ml bed volume,
equilibrated with buffer A (20 mM Hepes pH 7.0, 50 mM
5   NaCl, 50 mM ß-glycerophosphate, 1mM DTT, 0.2 mM EDTA,
0.02% NaN$_3$
and 0.1% NP-40)). All steps were conducted on ice or at
4°C. The flow-through was discarded and the column was
washed extensively with buffer A and then eluted in batch
10 with buffer B (Buffer A supplemented with 20 mM disodium
ATP, 20 mM EDTA and 0.5 M NaCl, pH 7.0). Ammonium
sulfate was added to the eluate to 50% (w/v) at 4°C over
the course of an hour, whereupon the suspension was
centrifuged at 10,000 x g for 45 minutes at 4°C. The
15 pellet was recovered, dissolved in buffer C (Buffer A
supplemented with 10 mM MnCl$_2$, pH 8.0) and desalted on
Sephadex G-25 that had been equilibrated with buffer C.
The desalted protein was applied to a column of Cibacron
Blue 3GA agarose (Type 3000) that had been equilibrated
20 with buffer C. The flow-through was discarded, the
column was washed extensively with buffer C, and eluted
in batch with buffer D (Buffer A supplemented with 20 mM
EDTA and 0.15 M NaCl, pH 8.0). The eluate was diluted
1:1 with buffer E (Buffer A with no added NaCl, pH 8.0)
25 and applied to phosphocellulose that had been
equilibrated with buffer F (Buffer A at pH 8.0). the
phosphocellulose was washed extensively and eluted in
batch with 0.6 M NaCl in buffer F. The eluate was
precipitated with trichloroacetic acid (10% final),
30 washed with acetone and solubilized in SDS sample buffer.
Proteins were resolved by SDS PAGE in 8% polyacrylamide,
blotted to nitrocellulose and visualized with Ponceau S.
The band corresponding to the autophosphorylation
activity of the 90 kD kinase was excised and digested

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with trypsin.   Tryptic peptides were resolved by HPLC and
microsequenced.

<u>Autophosphorylation of a 90 kD Kinase</u>

Jurkat cells were treated with 50 μM forskolin for
5 30 minutes and nuclear extracts of forskolin-stimulated
or control cells were fractionated on phosphocellulose.
Proteins were separated by polyacrylamide gel
electrophoresis in sodium dodecyl sulfate (SDS-PAGE),
transferred to nitrocellulose, denatured in guanidine
10 hydrochloride and assayed after renaturation.   The assay
revealed one major and two minor autophosphorylating
kinases.   The major activity, a multiplet with an
apparent mobility of 90 kD (spanning 84.5 - 92.7 kD), was
greater in extracts from forskolin-treated Jurkat cells
15 than in extracts from control cells (Fig. 1A).
Phosphocellulose chromatography resolved the multiplet
into forms of slightly lower and higher apparent
mobility, which we interpret to arise from differences in
protein phosphorylation.   The best separation and
20 recovery of forms was obtained by eluting the column
first with 0.3 M NaCl and then with 1.0 M NaCl.

Autophosphorylation of the species that eluted at
0.3 M NaCl increased sharply within 6 minutes of
stimulation and gradually declined through 30 minutes
25 (Fig. 1B).   Autophosphorylation of the species that
eluted at higher salt did not respond significantly to
forskolin stimulation.   If extracts were prepared without
the phosphatase inhibitors sodium vanadate (1 mM) and β-
glycerolphosphate (50 mM) (Yu et al., 1987), or if
30 extracts were briefly warmed to 37°C in the presence of 5
mM magnesium chloride, the 90 kD autophosphorylation
activity from stimulated cells was reduced to the levels
in control cells, whereas other species (e.g., 60 kD)
were unchanged.   Taken together, these results indicate
35 that this kinase is reversibly phosphorylated and

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increases its autophosphorylation in response to an
agonist of protein kinase A.

Other agents that affect Jurkat cell metabolism,
such as phytohemagglutinin, phorbol myristate acetate,
5   calcium ionophore A23187, serum and, interleukin-2, alone
or in combination, had little or no effect on the
autophosphorylation activity of the 90 kD multiplet in
Jurkat cells or peripheral blood lymphocytes over one
hour or over four days. Other conditions, such as
10 herpesvirus infection, heat shock, or exposure to
hydrogen peroxide, sodium periodate, sodium vanadate or
cadmium ion, were similarly ineffective. However,
interleukin-1α treatment (CHO cells starved of serum and
then treated with interleukin-1α at 10 ng/ml for 5, 10,
15 20, or 30 min) did result in significant stimulation of
90 kD kinase activity.

The 90 kD kinase appears to be ubiquitous, as we
have found well-resolved multiplets of similar apparent
molecular mass in human epithelial carcinoma (HeLa),
20 human epidermoid carcinoma (A431), human T-cell leukemia
(Jurkat), Epstein-Barr virus-transformed human B-cell
(32D cl3), human T-cell lymphoma (HUT78), mouse embryo
fibroblast and adipocyte (3T3-L1), mink lung (MvlLu),
Chinese hamster ovary (CHO), African green monkey kidney
25 (CV-1 and COS-7) cell lines, although the activities
differed in their chromatographic behavior on
phosphocellulose and varied slightly in their apparent
mobility. Depending on the cell type, up to ten
additional autophosphorylating species, with apparent
30 molecular masses as low as >20 kD, were also detectable.
It is not known whether these activities arise from
transcripts of homologous genes, or whether they are
unrelated to each other. In some cell lines, such as
A431, an 84 kD multiplet autophosphorylation activity
35 (78.4 - 89.2 kD) was both serum-inducible in serum-

- 27 -

starved cells and forskolin-inducible, and required
higher salt concentrations to elute from phosphocellulose
(Fig. 1C). Neither insulin nor glucagon treatment of
3T3-L1 adipocytes increased this autophosphorylation
5 activity, nor did forskolin or platelet-derived growth
factor treatment of CHO cells, nor did fibroblast growth
factor treatment of 3T3-L1 fibroblasts, nor did epidermal
growth factor or heat shock treatments of A431 cells
(Fig. 1C). These changes to autophosphorylation activity
10 were remarkably stable. Major differences in activity
between kinase prepared from control or stimulated cells
reproducibly survived cell lysis, fractionation, boiling
in SDS, gel electrophoresis, electroblotting,
denaturation in guanidine hydrochloride, renaturation
15 overnight in buffer at 4°C and exposure to 1 M sodium
hydroxide.

The blotted, renatured kinases were immobilized
and not in contact with potential protein substrates in
solution, so only autophosphorylation was observable.
20 These activities represent autophosphorylations, not
merely binding of ATP to renatured proteins because when
blots were probed in parallel with $[\alpha-^{32}P]ATP$ and $[\gamma-^{32}P]ATP$, only background radioactivity was associated with
the membranes probed with $[\alpha-^{32}P]ATP$, whereas discrete
25 bands were revealed with the membranes probed with $[\gamma-^{32}P]ATP$. The renatured kinase required magnesium ion for
activity. Blots were routinely incubated in 30 mM
magnesium chloride; manganese ion, which was tested from
1 mM to 30 mM, was a poor substitute.
30   Physical properties of the 90 kD kinase
The autophosphorylation activities present in
nuclear and cytosolic extracts of HeLa cells were
compared. A homogenate of HeLa cells was prepared in the
presence of phosphatase inhibitors as described above and
35 centrifuged at 100,000 x g for one hour at 4°C. The

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protein concentration of the supernatant (S-100) was
determined and equal amounts of protein from the S-100
and nuclear extract (also cleared of particulate residue)
were subjected to SDS-PAGE. Proteins were electroblotted
5 to nitrocellulose, renatured and probed with [γ-^{32}P]ATP.
As shown in Fig. 2, an autophosphorylation signal (91.0 -
98.8 kD) in nuclear extract is virtually absent from
cytosolic extract.
   Other autophosphorylation activities resolved on this
10 blot, however, appear to be roughly equally represented
in nuclear and cytosolic fractions (70.9, 64.3, 52.8 and
37.9 kD). This result suggested that the 90 kD kinase is
predominantly nuclear in localization.
          In vitro phosphorylation of protein and peptide
15 _substrates by the 90 kD kinase (RING3-related kinase)_
          Phosphocellulose-purified, renatured 90 kD kinase
was tested for its substrate specificity with several
different protein and peptide substrates: S6 peptide
(RRLSSRA (SEQ ID NO: 14); Pelech et al., 1986), MAP
20 kinase substrate peptide (APRTPGGRR (SEQ ID NO: 15);
Sanghera et al. 1990), casein kinase II substrate
(RRREEETEEE (SEQ ID NO: 16); Kuenzel and Krebs, 1985)
protein kinase C serine-25 substrate (RFARKGSLRQKNV (SEQ
ID NO: 17); House and Kemp, 1987), calmodulin-dependent
25 kinase substrate (PLSRTLSVSS-NH$_2$ (SEQ ID NO: 18); Pearson
et al., 1985), smooth muscle myosin light chain kinase
substrate (LLRPQRATSNVFS-NH$_2$ (SEQ ID NO: 19); Kemp et
al., 1983), Kemptide, a substrate for protein kinase A
(LRRASLG (SEQ ID NO: 20); "Kemptide," Kemp et al., 1983),
30 Raytide (a tyrosine kinase substrate), histones H1, H2,
H2b, H3 and myelin basic protein. The results are shown
in Table I:

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Table 1
In vitro phosphorylation of peptides by renatured 90 kD kinase

peptide substrate	$[^{32}P]$cpm	(SE)
none	1205	±465
S6 peptide	3005	±535
MAP kinase substrate peptide	1443	±210
casein kinase II peptide	1591	±482
protein kinase C peptide	2056	±465
calmodulin-dependent kinase peptide	2330	±866
myosin light chain kinase peptide	58,690	±870
Kemptide	44,430	±5258
Kemptide[1]	>1,000,000	ND
Raytide	4981	±377
Raytide[2]	322,920	ND

[1] Phosphorylated with the catalytic subunit of protein kinase A
[2] Phosphorylated with recombinant p43$^{v\text{-}abl}$.

The partially purified kinase phosphorylated the
peptide substrates for myosin light chain kinase and
protein kinase A, and myelin basic protein. Myelin basic
protein is a useful *in vitro* substrate for MAP kinases,
but the MAP kinase substrate peptide (which includes the
amino acid residues that MAP kinases ordinarily
phosphorylate) was not phosphorylated here. The
catalytic subunit of protein kinase A was used as a
positive control for Kemptide phosphorylation and
recombinant p43$^{v\text{-}abl}$ was used as a positive control for
Raytide phosphorylation. These results suggest that the
90 kD kinase has a narrow substrate specificity and with
respect to its substrate preferences in unlike other
known kinases.

HeLa nuclear extracts were fractionated on
phosphocellulose as described. Proteins that eluted at
0.3 M and 1.0 M NaCl were blotted to nitrocellulose,
renatured and tested for their ability to phosphorylate
myelin basic protein that was supplied in solution with
magnesium chloride and $[\gamma\text{-}^{32}P]$ATP. The kinase that eluted
at the lower salt concentration, which is the inducible
form in Jurkat cells and which has a slightly slower

- 30 -

apparent mobility (Fig. 1A), showed greater substrate-directed phosphorylation activity than the species that eluted at the higher salt concentrations (Fig. 4). This result suggests that induced autophosphorylation activity
5 is linked to increased substrate-directed phosphorylation activity. To refine this result, HeLa nuclear extract was more narrowly fractionated on phosphocellulose. Eluates were blotted to nitrocellulose and renatured either for autophosphorylation or for phosphorylation of
10 myelin basic protein supplied in solution. Autophosphorylation of the slower mobility form was correlated with phosphorylation of myelin basic protein, whereas autophosphorylation of the faster mobility form was not (Figs. 5A and 5B).
15   Without being bound by any particular theory, it appears that the 90 kD kinase is phosphorylated by a kinase to generate a hyperphosphorylated form that is the active participant in signal transduction.

Phosphoaminoacid Analysis
20   The 90 kD kinase was blotted to a polyvinyldifluoride membrane and autophosphorylated with $[\gamma\text{-}^{32}P]ATP$. The immobilized protein was hydrolyzed in 6N HCl, and the phosphoaminoacids were separated by electrophoresis on silica. Autoradiography revealed that
25 radioactivity was associated in equal measure with ninhydrin-visualized standards for phosphoserine (pS) and phosphothreonine (pT). No radioactivity was associated with a phosphotyrosine (pY) standard (Fig. 6A). Myelin basic protein that had been phosphorylated in vitro with
30 renatured 90 kD kinase was solubilized in SDS and resolved by PAGE. Phosphorylated myelin basic protein was visualized with Coomassie brilliant blue, excised from the gel and acid-hydrolyzed as before. Radioactivity was associated only with pS, not with pT or
35 pY (Fig. 6B). As a positive control for tyrosine

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phosphorylation, recombinant p43^{v-abl} was incubated with
myelin basic protein under the same conditions. In this
case, pY alone was observed (Fig. 6B).

Purification and microsequencing of the 90 kD

5 Kinase

The 90 kD nuclear kinase shares some of the
characteristics of signal-transducing kinases such as
pp90^{rsk}, pp42^{mapk}/pp44^{mapk} and protein kinase C (Chen et
al., 1992; Chung et al., 1991; Leach et al., 1989).
10 Western blots of partially purified 90 kD kinase were
probed with rabbit polyclonal antibodies to RSK, MAP
kinase and protein kinase C. Protein kinase C from rat
brain was used as a positive control. These antisera
displayed no detectable immunoreactivity with the
15 partially purified kinase above background.

From these results, we suspected that this 90 kD
protein had not been studied previously. We therefore
undertook to purify it and obtain microsequence
information. HeLa nuclear extract was prepared in the
20 presence of 50 mM ß-glycerophosphate and 1 mM sodium
vanadate. The autophosphorylation activity provided the
assay for purification. The partially purified kinase
was resolved by SDS-PAGE, blotted to nitrocellulose and
digested with trypsin. Tryptic peptides were separated
25 by HPLC and two peptides were microsequences. The first
of these (DSNPDEIEIDFETLKPTTL) (SEQ ID NO: 21) was used
to probe the GenBank database; a statistically
significant match was obtained to the female sterile
homeotic (fsh) gene of Drosophila (DROFSHB; ATCC
30 accession number M23222, Haynes et al., 1989). There
were 18/19 identities (95% match, with a S/T substitution
at residue 17). A statistically significant match was
also obtained with the RING3 gene of humans 9HUMRING3;
ATCC accession number M80613, Beck et al., 1992) (17/19
35 identities (89% match) with a E/D substitution at

- 32 -

position 5 in addition to the S/T substitution of fsh).
The second peptide (AVHEQLAALSQAP) (SEQ ID NO: 22) gave a
statistically significant match to RING3 (12/13
identities (92%) with a A/G substitution at position 12),
5 but did not match fsh. The first peptide lies within the
C-terminal region of highest homology between fsh and
RING3, whereas the second peptide lies outside of this
region, in the N-terminal direction (Beck et al., 1992).
These results were surprising because neither RING3 nor
10 fsh have been reported to be kinases. However,
inspection of the protein sequence of these genes reveals
several correctly spaced amino acid motifs that are
consistent with the genes encoding a protein kinase
(Hanks et al., 1988; Hanks, S., 1991), although of
15 unusual organization. It was important to determine
whether the gene products exhibit kinase activity.

       Clones that encode the cDNAs for fsh and RING3
were obtained from the authors of the original published
reports. The cDNA for fsh was a set of overlapping
20 clones, one of which, e5.16 (Haynes et al., 1989),
included the putative catalytic region of interest. This
cDNA contained a site for NcoI that provided an in-frame
methionine at position 785 of the open reading frame,
almost 100 amino acids upstream from the putative GXGXXG
25 (SEQ ID NO: 23), and ran through the stop codon at amino
acid 1107. The fragment was fused in-frame to the 5'
untranslated region of the β-globin gene and subjected to
in vitro transcription/translation in both rabbit
reticulocyte and wheat germ extracts as described.
30 Products were separated by SDS-PAGE, blotted to
nitrocellulose, denatured, renatured and probed with [γ-
^{32}P]ATP as described above. No radioactivity was
associated with renatured bands in excess of the signals
seen in extracts primed with control viral mRNA. Several
35 kinases in the translation reaction were capable of

- 33 -

renaturation by this assay and may have obscured signals
from the kinase in question. The fsh fragment was also
expressed as a histidine-tagged protein, a T7 antibody-
tagged protein and as a glutathione-S-transferase (GST)
5   fusion protein. The bacterially expressed fusion protein
showed little or no activity. The same was true for
fusion constructs of RING3, with had a convenient NcoI
site to yield an in-frame methionine at amino acid 347 of
the open reading frame, just over 200 residues upstream
10 from the putative GXGXXG (SEQ ID NO: 23) and continued
through the run of serines to the stop codon at 755. The
failure of these bacterially translated cDNAs to
demonstrate kinase activity *in vitro* can probably be
ascribed to the usual shortcomings with post-
15 translational processing in prokaryotes.

      Recombinant RING3 protein was incubated with HeLa
nuclear extract and ATP, repurified and assayed again.
The protein now possessed kinase activity. This result
implies the existence of a kinase in nuclear extract.
20 For mammalian overexpression, COS cells were transiently
transfected with a CMV expression vector carrying RING3
cDNA. Nuclear extracts were prepared from these
transfected cells and fractionated by phosphocellulose
chromatography, which we found could resolve ectopically-
25 expressed from endogenous kinase activity. We believe
that the distinct chromatographic behavior of ectopic and
endogenous kinase is due to differences in their
phosphorylation. These results strongly suggest that
RING3 cDNA encodes renaturable 90 kDa nuclear kinase
30 activity.

      For these studies, RING3 cDNA (clone CEM32, a 4 kb
insert in CDM8; Beck et al., 1992) was propagated in
MC1061/P3 (Invitrogen). An NcoI - EcoRI fragment
(nucleotides 2214-3640) was excised from RING3 and
35 ligated into the BamHI and EcoRI sites of pGEX-2T for

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overexpression (Lin and Green, 1991). Rabbit polyclonal
antibody was raised against purified GST fusion protein.
For production of purified antibody, rabbit immune sera
were incubated with GST-agarose to remove antibodies
5   against GST epitopes. Polyclonal antibodies against
RING3 epitopes were then purified by antigen affinity
chromatography on Affigel columns of the purified GST
fusion protein.

        For construction of a eukaryotic overexpression
10 vector, RING3 cDNA was obtained by double-stranded
polymerase chain reaction (PCR) amplification of clone
CEM32 with a forward primer (5'-
CGCCGCGGATCCATGGCTTCGGTGCCTGCT-3') (SEQ ID NO: 24) that
engineered a 5' BamHI site at the amino-terminal
15 methionine of the coding sequence and a reverse primer
(5'-GGCTGGGAATTCAATGTT-3') (SEQ ID NO: 25) that employed
a native EcoRI site in the 3' untranslated region. The
BamHI-EcoRI fragment from PCR was ligated into the BamHI
and EcoRI sites in the multicloning site of pcDNA(I)
20 (Invitrogen). For construction of a bacterial
overexpression vector, the BamHI-EcoRI fragment was
ligated in-frame into the same sites of RSETA
(Invitrogen), to fuse six histidines to the N-terminus of
RING3. Clones were verified by dideoxy sequencing.
25 Histidine-tagged recombinant protein was purified on Ni^+-
agarose (Qiagen), and phosphorylated by incubation for 30
min at 30°C with HeLa nuclear extract in Buffer A that
contained with 10 mM ATP and 5 mM $MgCl_2$ but no Na
vanadate. Phosphorylated protein was repurified on Ni^+-
30 agarose and assayed by in-gel kinase assay (Gotoh et al.,
1990).

        The GST fusion proteins with the fsh and RING3
fragments were purified from lysates of induced bacteria,
purified on glutathione agarose and injected into rabbits
35 for the production of antibody. The fusion constructs

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were of the correct electrophoretic mobility on SDS gels.
Immune sera were screened for the ability to
immunoprecipitate the original kinase activity from HeLa
nuclear extract. Both immune sera to GST-fsh and to GST-
5 RING3 immunoprecipitated the renaturable
autophosphorylation activity with apparent molecular
weight 90 kD, whereas none of the preimmune sera
immunoprecipitated this activity. This result strongly
supports the interpretation that fsh and RING3 are
10 kinases, and that the microsequenced peptides are derived
from a HeLa gene product that is homologous to the
Drosophila and human T-cell cDNAs.

Rabbit immune sera were incubated with GST-agarose
to remove antibodies against GST epitopes. Polyclonal
15 antibodies against RING3 and fsh epitopes were then
purified by antigen affinity chromatography on Affigel
columns of each purified GST fusion protein. Purified
antibodies were assayed by immunoblot. Indirect
immunofluorescence of HeLa cells with purified polyclonal
20 antibody and with fluorescein-conjugated second antibody
shows that RING3 is localized to the nucleus (Fig. 8).
This result is in agreement with Fig. 2, which suggested
that the kinase is exclusively nuclear, and with the
presence of a putative nuclear localization signal in the
25 open reading frame of the RING3 cDNA. Antibody was
excluded from nucleoli, consistent with patterns of
nuclear staining for proteins that are involved in
transcription.

Example 2
Biochemical Assay for Leukemia

30 The above-described assay (Fig. 1A) for 90 kD
kinase activity can be used to diagnose leukemia. The
following experiment is one example of a suitable
diagnostic assay.

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For each patient assayed cell extracts were
prepared from 10^7 PBLS. The proteins were separated by
SDS-PAGE, renatured and assayed for 90 kD kinase
autophosphorylation activity as described above (Fig.
5 1A). The results of this analysis are presented in Fig.
9A. The following patients were used: a normal 34 year-
old male (lane 1); a 69 year-old male diagnosed with
chronic lymphocytic leukemia (> 10^6 mature T-cells/μl,
lymphostasis, immune suppression) (lane 2); a 4 year-old
10 female diagnosed with acute lymphocytic leukemia in
proliferative blast stage (lane 3); a 15 year-old male
diagnosed with acute lymphocytic leukemia in
proliferative blast stage (lane 4); the same 15 year-old
male after chemotherapy (lane 5). The results show that
15 there is significant 90 kD kinase activity in sample from
patients with active leukemia, but not in normal patients
or those in remission. These results are representative
of ten normal controls and ten acute lymphocytic leukemia
patients (> 50,000 blasts/μl in each case) and ten normal
20 control patients.

These results demonstrate that 90 kD kinase
activity can be used to diagnose leukemia and detect the
presence of leukemic cells. While 90 kD kinase activity
was increased in acute lymphocytic leukemia patients, the
25 expression of the 90 kD kinase, as determined by
immunoblotting with anti-RING3 antibody, was
approximately the same in acute lymphocytic leukemia
patients and normal controls.

The level of 90 kD kinase activity may be used as
30 an indication of the stage of leukemic progression.
Vonken et al. (1992) and Griffiths et al. (1992) describe
a murine model of leukemia which permits study of early
stages of leukemia as well as its progression. This
model is useful for adapting 90 kD kinase activity assays
35 for monitoing disease progression. By measuring the

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activity of 90 kD kinase it is possible to determine the degree of disease progression.

As an alternative method for measuring 90 kD kinase, anti-RING3 antibody can be used to precipitate 90 kD kinase. The immune complexes are then washed, solubilized with SDS and autophosphorylation is assayed as described above.

When autophosphorylation activity was measured in cell extract, a prominent 50 kD band was observed in the samples taken from the normal control and the patient in remission, but not the leukemic patients. The absence of activity might be able to serve as an additional indication of a leukemic condition.

Assays for 90 kD Kinase Activity

Any means for measuring the activity of the 90 kD kinase can be used as an assay for leukemia. In addition to the assays described above and in Figs. 1A, 9A and 9B, the level of 90 kD kinase activity can be determined by measuring the phosphorylation of a natural or synthetic 90 kD kinase substrate. For example, an ELISA-based assay can be used. In one such assay substrate peptide, immobilized on a solid support, is incubated with a 90 kD kinase-containing sample (a crude extract or a partially purified sample) in the presence of ATP (added if needed). Phosphorylated substrate is then detected by an antibody which binds the phosphorylated, but not the non-phosphorylated, substrate followed by treatment with an enzyme-conjugated secondary antibody and enzyme substrate.

It sould be understood that it may not be necessary to carry out an autophosphorylation reaction in order to dectec active 90 kD kinase. The 90 kD kinase in the leukemic (or pre-leukemic) cells may be substantially autophosphorylated.

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In an alternative assay, 90 kD kinase in a test
sample is substantially or partially purified by
immunoprecipitation using an anti-90 kD kinase antibody.
Substrate and $[\gamma^{32}P]$-ATP (or other appropriate label) are
5   then incubated with the partially purified 90 kD kinase
in an appropriate buffer (generally containing ATP, DTT,
$MgCl_2$, and other components). An aliquot of the reaction
mixture is transferred to a solid support (e.g., a
phosphocellulose filter) and the radioactivity is
10 detected after washing (e.g., with 1% phosphoric acid) to
remove free $[\gamma^{32}P]$-ATP.   In any assay it is important
to eliminate other kinases which might significantly
interfere with the ability to detect phosphorylation by
the 90 kD kinase.   Interference can be avoided by
15 employing a substrate which is not significantly
phosphorylated by other kinase present in the sample
(e.g., the cell extract). Alternatively, the 90 kD
kinase present in the sample can be partially purified to
substantially eliminate interfering kinases.   This
20 purification can be accomplished by any standard
procedure, including affinity purification with
antibodies selective for the 90 kD kinase (or RING3).
For purposes of partial or complete purification it is
not required that the anti-90 kD antibody be selective
25 for the autophosphorylated form of the 90 kD kinase.

Anti-90 kD kinase Antibodies

Human 90 kD kinase (or immunogenic fragments or
analogues) may be used to raise antibodies useful in the
invention; such polypeptides may be produced by
30 recombinant or peptide synthetic techniques (see, e.g.,
Solid Phase Peptide Synthesis, infra; Ausubel et al.,
1994). The peptides may be coupled to a carrier protein,
such as KLH as described in Ausubel et al., 1994. The
KLH-peptide is mixed with Freund's adjuvant and injected
35 into guinea pigs, rats, or preferably rabbits.

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Antibodies may be purified by peptide antigen affinity chromatography.

Monoclonal antibodies may also be prepared using the 90 kD kinases described above and standard hybridoma
5   technology (see, e.g., Kohler et al., 1975; Kohler et al., 1976a; Kohler et al., 1976b; Hammerling et al., 1981; Ausubel et al., 1994).

Once produced, polyclonal or monoclonal antibodies are tested for specific 90 kD kinase recognition by
10 Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., 1994). Antibodies which specifically recognize the 90 kD kinase are considered to be useful in the invention; such antibodies may be used, e.g., in an immunoassay to monitor the level
15 of 90 kD kinase produced by a mammal (for example, to determine the amount or subcellular location of 90 kD kinase).

Preferably, antibodies of the invention are produced using fragments of the 90 kD kinase which appear
20 likely to be antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR and cloned into the pGEX expression vector (Ausubel et al., 1994). Fusion proteins are expressed in *E. coli*
25 and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., 1994). To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three such fusions are generated for each protein, and each fusion is injected
30 into at least two rabbits. Antisera are raised by injections in a series, preferably including at least three booster injections.

Antisera is cleared of anti-GST antibodies using GST immobilized on a glutathione column, and the antisera
35 are checked by ELISA for titer and specificity, using GST

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fusion proteins as controls. Antisera is also checked
for its ability to immunoprecipitate *in vitro* translated
90 kD kinases or control proteins, such as glucocorticoid
receptor, CAT, or luciferase. Western blots of total or
5   nuclear versus cytoplasmic fractionated HeLa cell
proteins are also probed with the antisera to assess
specificity and to characterize subcellular
compartmentalization. In these and other immunologic
assays, specificity is confirmed by the specific
10 competition with the GST fusion protein.

Once the specificity of an antiserum is confirmed,
it may be used in any standard indirect
immunofluorescence procedure to determine the amount or
subcellular distribution of 90 kD kinase in a particular
15 cell type.

In many applications anti-RING3 antibodies,
produced as described herein or by other standard
techniques may be useful for in the methods of the
invention for the same purposes as anti-90 kD kinase
20 antibodies.
Anti-90 kD Kinase Antibodies Specific for
Autophosphorylated 90 kD Kinase

Antibodies which are selective for the
phosphorylated form of 90 kD kinase (particularly the
25 form created by autophosphorylation) are particularly
useful. To be useful in diagnostic assays, the antibody
must be selective for the site where autophosphorylation
occurs. This is because the 90 kD kinase may be
phosphorylated by other kinases. Antibodies which
30 recognize these phosphorylation sites that are not
autophosphorylation sites are far less likely to be
useful for detecting activated 90 kD kinase.

Appropriate antibodies will not substantially bind
non-autophosphorylated forms of 90 kD kinase. Such
35 antibodies can be prepared by immunization with the

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appropriate phosphopeptide derived from the 90 kD kinase
(or RING3). A suitable phosphopeptide can be prepared by
digesting phosphorylated protein and then isolating and
purifying one or more suitable phosphorylated
5   polypeptides, which peptides were created by
autophosphorylation. Alternatively, a synthetic peptide
based on the phosphorylation site of the 90 kD kinase (or
RING3) can be phosphorylated *in vitro*, e.g., by the 90 kD
kinase (or other kinase which phosphorylates the relevant
10 site, e.g., protein kinase C or casein kinase II or
protein kinase A may phosphorylate the relevant site on
the 90 kD kinase), and then used for immunization. The
generation of antibodies which selectively bind the
autophosphorylated, but not the non-autophosphorylated
15 form of the 90 kD kinase can be carried out according to
procedures similar to those which have been used by
others to prepare other phosphoprotein selective
antibodies. For example, Ginty et al. (1993) describes
the creation of a polyclonal antibody which selectively
20 binds the phosphorylated, but not the non-phosphorylated,
form of rat cyclic AMP response element binding protein.
        Antibodies which are selective for active (or
inactive) 90 kD kinase may be used in any standard
immunoassay format (e.g., ELISA, Western blot, or RIA
25 assay) to measure 90 kD kinase polypeptide levels; again
comparison would be to normal levels of active (or
inactive) 90 kD kinase, and a change (increase) in 90 kD
kinase expression or activation would be indicative of a
leukemic or pre-leukemic condition. Examples of
30 immunoassays are described, e.g., in Ausubel et al.,
1994. Immunohistochemical techniques may also be
utilized for 90 kD kinsase detection. For example, a
tissue sample may be obtained from a patient, and a
section stained for the presence of 90 kD kinase using an
35 anti-90 kD kinase antibody and any standard detection

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system (e.g., one which includes a secondary antibody
conjugated to horseradish peroxidase). General guidance
regarding such techniques can be found in, e.g., Bancroft
and Stevens (*Theory and Practice of Histological*
5 *Techniques*, Churchill Livingstone, 1982) and Ausubel et
al. 1994).

Identification of Molecules that Modulate 90 kD Kinase
Expression or Activity

Identification of the 90 kD kinase facilitates the
10 identification of molecules which increase or decrease
its expression or activity. According to one approach,
candidate molecules (e.g., peptide or non-peptide
molecules found, e.g., in a cell extract, mammalian
serum, or growth medium on which mammalian cells have
15 been cultured or as a part of a small molecule screening
library) are added at varying concentrations to the
culture medium of cells (e.g., HeLa or COS cells) which
express 90 kD kinase mRNA. Expression of the 90 kD
kinase is then measured by standard Northern blot
20 analysis (Ausubel et al., 1994) using a 90 kD kinase (or
RING3) cDNA (or cDNA fragment) as a hybridization probe.
The level of 90 kD kinase expression in the presence of
the candidate molecule is compared to the level measured
for the same cells in the same culture medium but in the
25 absence of the candidate molecule. Alternatively,
activity of the 90 kD kinase may be measured (e.g., using
one of the assays described herein). The level of 90 kD
kinase activity in the presence of the candidate molecule
is compared to the level measured for the same cells in
30 the same culture medium but in the absence of the
candidate molecule.

Candidate modulators may be purified (or
substantially purified) molecules or may be one component
of a mixture of compounds (e.g., an extract or
35 supernatant obtained from cells; Ausubel et al., 1994).

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In a mixed assay, 90 kD kinase expression (or activity)
is tested against progressively smaller subsets of the
candidate compound pool (e.g., produced by standard
purification techniques, e.g., HPLC or FPLC) until a
5   single compound is finally demonstrated to modulate the
the 90 kD kinase activity. Candidate 90 kD kinase
modulators include peptide as well as non-peptide
molecules.

90 kD kinase Expression

10   In general, 90 kD kinase according to the
invention may be produced by transformation of a suitable
host cell (e.g., COS cells or HeLa cells) with all or
part of a 90 kD kinase-encoding cDNA fragment in a
suitable expression vehicle.

15   Those skilled in the field of molecular biology
will understand that any of a wide variety of expression
systems may be used to provide the recombinant protein.
The precise host cell used is not critical to the
invention. The 90 kD kinase may be produced in a
20 prokaryotic host (e.g., *E. coli*) or in a eukaryotic host
(e.g., *Saccharomyces cerevisiae* or mammalian cells, e.g.,
COS 1, NIH 3T3, or HeLa cells). Such cells are available
from a wide range of sources (e.g., the American Type
Culture Collection, Rockland, MD; also, see, e.g.,
25 Ausubel et al., 1994). The method of transformation or
transfection and the choice of expression vehicle will
depend on the host system selected. Transformation and
transfection methods are described, e.g., in Ausubel et
al., *supra*; expression vehicles may be chosen from those
30 provided, e.g., in *Cloning Vectors*: A Laboratory Manual
(Pouwels et al., 1985, Supp. 1987).

        Other suitable expression systems include
Baculovirus and Vaccinia virus systems as described,
e.g., in Ausubel et al., *supra*.

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One preferred expression system is the mouse 3T3
fibroblast host cell transfected with a pMAMneo
expression vector (Clontech, Palo Alto, CA). pMAMneo
provides: an RSV-LTR enhancer linked to a dexamethasone-
5   inducible MMTV-LTR promotor, an SV40 origin of
replication which allows replication in mammalian
systems, a selectable neomycin gene, and SV40 splicing
and polyadenylation sites. DNA encoding a 90 kD kinase
would be inserted into the pMAMneo vector in an
10   orientation designed to allow expression. The
recombinant 90 kD kinase would be isolated as described
below. Other preferable host cells which may be used in
conjunction with the pMAMneo expression vehicle include
COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and
15   CCL 61, respectively). Cytomegalovirus promoter-based
expression systems may also be useful.

Alternatively, the 90 kD kinase can be produced by
a stably-transfected mammalian cell line. A number of
vectors suitable for stable transfection of mammalian
20   cells are available to the public, e.g., see Pouwels et
al., supra; methods for constructing such cell lines are
also publicly available, e.g., in Ausubel et al., 1994.
In one example, cDNA encoding the 90 kD kinase is cloned
into an expression vector which includes the
25   dihydrofolate reductase (DHFR) gene. Integration of the
plasmid and, therefore, the 90 kD kinase-encoding gene
into the host cell chromosome is selected for by
inclusion of 0.01-300 μM methotrexate in the cell culture
medium (as described in Ausubel et al., 1994). This
30   dominant selection can be accomplished in most cell
types. Recombinant protein expression can be increased
by DHFR-mediated amplification of the transfected gene.
Methods for selecting cell lines bearing gene
amplifications are described in Ausubel et al., 1994;
35   such methods generally involve extended culture in medium

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containing gradually increasing levels of methotrexate.
DHFR-containing expression vectors commonly used for this
purpose include pCVSEII-DHFR and pAdD26SV(A) (described
in Ausubel et al., 1994). Any of the host cells

5 described above or, preferably, a DHFR-deficient CHO cell
line (e.g., CHO DHFR⁻cells, ATCC Accession No. CRL 9096)
are among the host cells preferred for DHFR selection of
a stably-transfected cell line or DHFR-mediated gene
amplification.

10   Once the recombinant 90 kD kinase is expressed, it
is isolated, e.g., using affinity chromatography. In one
example, an anti-90 kD kinase antibody (e.g., produced as
described herein) may be attached to a column and used to
isolate the 90 kD kinase. Lysis and fractionation of 90

15 kD kinase-harboring cells prior to affinity
chromatography may be performed by standard methods (see,
e.g., Ausubel et al., 1994). Alternatively, a 90 kD
kinase fusion protein, for example, a 90 kD kinase-
maltose binding protein, a 90 kD kinase-β-galactosidase,·

20 or a 90 kD kinase-trpE fusion protein, may be constructed
and used for 90 kD kinase isolation (see, e.g., Ausubel
et al., 1994; New England Biolabs, Beverly, MA).

        Once isolated, the recombinant protein can, if
desired, be further purified, e.g., by high performance

25 liquid chromatography (see, e.g., Fisher, *Laboratory
Techniques In Biochemistry And Molecular Biology*, eds.,
Work and Burdon, Elsevier, 1980).

        Polypeptides of the invention, particularly short
90 kD kinase fragments, can also be produced by chemical

30 synthesis (e.g., by the methods described in *Solid Phase
Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical
Co., Rockford, IL).

        These general techniques of polypeptide expression
and purification can also be used to produce and isolate

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useful fragments or analogs of the 90 kD kinase
(described herein).

Other Embodiments

In other embodiments, the invention includes a
5 substantially pure preparation of 90 kD kinase.
Preferably, the 90 kD kinase includes an amino acid
sequence substantially identical to that of human 90 kD
kinase.

The invention also features purified DNA (for
10 example, cDNA) which includes a sequence encoding a 90 kD
kinase, preferably encoding a human 90 kD kinase; a
vector and a cell which includes a purified DNA of the
invention; and a method of producing a recombinant 90 kD
kinase involving providing a cell transformed with DNA
15 encoding 90 kD kinase positioned for expression in the
cell, culturing the transformed cell under conditions for
expressing the DNA, and isolating the recombinant 90 kD
kinase. The invention further features recombinant 90 kD
kinase produced by such expression of a purified DNA of
20 the invention.

By "90 kD kinase polypeptide" is meant a
polypeptide which can autophosphorylate and is homologous
to a polypeptide fragment of the 90 kD kinase.
Preferably, such a polypeptide has an amino acid sequence
25 which is most preferably 95% or even 99% identical to the
amino acid sequence of human 90 kD kinase.

For polypeptides, the length of comparison
sequences will generally be at least 20 amino acids,
preferably at least 30 amino acids, more preferably at
30 least 50 amino acids, and most preferably 100 amino
acids.

Homology is typically measured using sequence
analysis software (e.g., Sequence Analysis Software
Package of the Genetics Computer Group, University of
35 Wisconsin Biotechnology Center, 1710 University Avenue,

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Madison, WI 53705). Such software matches similar
sequences by assigning degrees of homology to various
substitutions, deletions, substitutions, and other
modifications. Conservative substitutions typically
5   include substitutions within the following groups:
glycine, alanine; valine, isoleucine, leucine; aspartic
acid, glutamic acid, asparagine, glutamine; serine,
threonine; lysine, arginine; and phenylalanine, tyrosine.

By "protein" and "polypeptide" is meant any chain
10 of amino acids, regardless of length or post-
translational modification (e.g., glycosylation or
phosphorylation).

By "substantially pure" is meant a preparation
which is at least 60% by weight (dry weight) the compound
15 of interest, i.e., a 90 kD kinase. Preferably the
preparation is at least 75%, more preferably at least
90%, and most preferably at least 99%, by weight the
compound of interest. Purity can be measured by any
appropriate method, e.g., column chromatography,
20 polyacrylamide gel electrophoresis, or HPLC analysis.

By "purified DNA" is meant DNA that is not
immediately contiguous with both of the coding sequences
with which it is immediately contiguous (one on the 5'
end and one on the 3' end) in the naturally occurring
25 genome of the organism from which it is derived. The
term therefore includes, for example, a recombinant DNA
which is incorporated into a vector; into an autonomously
replicating plasmid or virus; or into the genomic DNA of
a prokaryote or eukaryote, or which exists as a separate
30 molecule (e.g., a cDNA or a genomic DNA fragment produced
by PCR or restriction endonuclease treatment) independent
of other sequences. It also includes a recombinant DNA
which is part of a hybrid gene encoding additional
polypeptide sequence.

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By "substantially identical" is meant an amino
acid sequence which differs only by conservative amino
acid substitutions, for example, substitution of one
amino acid for another of the same class (e.g., valine
5 for glycine, arginine for lysine, etc.) or by one or more
non-conservative substitutions, deletions, or insertions
located at positions of the amino acid sequence which do
not destroy the function of the protein (assayed, e.g.,
as described herein). Preferably, such a sequence is at
10 least 90%, more preferably 95%, and most preferably 99%
identical at the amino acid level to the sequence human
90 kD kinase. For nucleic acids, the length of
comparison sequences will generally be at least 50
nucleotides, preferably at least 60 nucleotides, more
15 preferably at least 75 nucleotides, and most preferably
110 nucleotides. A "substantially identical" nucleic
acid sequence codes for a substantially identical amino
acid sequence as defined above.

By "transformed cell" is meant a cell into which
20 (or into an ancestor of which) has been introduced, by
means of recombinant DNA techniques, a DNA molecule
encoding (as used herein) 90 kD kinase.

By "positioned for expression" is meant that the
DNA molecule is positioned adjacent to a DNA sequence
25 which directs transcription and translation of the
sequence (i.e., facilitates the production of 90 kD
kinase).

By "purified antibody" is meant antibody which is
at least 60%, by weight, free from the proteins and
30 naturally-occurring organic molecules with which it is
naturally associated. Preferably, the preparation is at
least 75%, more preferably at least 90%, and most
preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody which
35 recognizes and binds 90 kD kinase but which does not

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substantially recognize and bind other molecules in a
sample, e.g., a biological sample, which naturally
includes 90 kD kinase.

In another aspect the invention relates to a
5 method for regulating transcription of a gene of
interest. The method comprises modulating the activity
of a kinase that activates transcription of the gene and
is (i) substantially exclusively intranuclearly
localized; (ii) capable of autophosphorylation; (iii)
10 selectively bindable with antibodies raised against the
RING3 portion of GST-RING3; (iv) of a molecular weight of
from about 82.5 to about 92.7 kilodaltons; and (v)
includes peptide sequences Asp-Ser-Asn-Pro-Asp-Glu-Ile-
Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-Pro-Thr-Thr-Leu (SEQ ID
15 NO: 1) and Ala-Val-His-Glu-Gln-Leu-Ala-Ala-Leu-Ser-Gln-
Ala-Pro (SEQ ID NO: 2).

In this aspect, modulating the kinase activity can
comprise stimulating the phosphorylation activity of the
kinase, so as to prevent inappropriate transcription of a
20 regulated gene of interest. More particularly, the
kinase activates a transcription factor for initiating
transcription of the gene of interest. Alternatively,
modulating the kinase activity can comprise inhibiting
the phosphorylation activity of the kinase so as to
25 inhibit the transcription of the gene of interest. More
particularly, the kinase activates a transcription factor
for the gene of interest.

Another aspect of the invention provides a method
for regulating the transcription of a gene of interest in
30 a cell which comprises contacting said cell with a
compound that specifically binds with a cell surface
receptor so as to modulate by signal transduction the
activity of a kinase that: (i) is substantially
exclusively intranuclearly localized; (ii) is capable of
35 autophosphorylation; (iii) is selectively bindable with

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antibodies rais d against the RING3 portion of GST-RING3;
(iv) has a molecular weight of from about 82.5 to about
92.7 kilodaltons; and (v) includes peptide sequences Asp-
Ser-Asn-Pro-Asp-Glu-Ile-Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-
5   Pro-Thr-Thr-Leu (SEQ ID NO: 1) and Ala-Val-His-Glu-Gln-
Leu-Ala-Ala-Leu-Ser-Gln-Ala-Pro (SEQ ID NO: 2). In this
aspect, the compound stimulates a cell surface receptor
including those from the group consisting of peptide
growth factor receptors, steroid and other hormone
10 receptors, ion channel receptors, serpentine cell surface
receptors and immunophilins. This aspect also relates to
stimulating the signal transduction function of said
kinase by contacting said cell with forskolin or analog
or derivation thereof.
15   Also, lectin-binding receptors, antibody/antigen
receptors, cell contact receptors that are otherwise
involved in self/non-self or immune recognition or that
are encoded by the major histocompatibility complex, cell
adhesion receptors, steroid and other hormone receptors,
20 chemotactic receptors and receptors for biochemical
molecules, which molecules are involved in signal
transduction.
        In this aspect, regulating can comprise
stimulating the phosphorylation activity of the kinase so
25 as to either induce transcription or prevent inhibition
of transcription of the gene of interest. Alternatively
this aspect can include a method for inhibiting the
phosphorylation activity of the kinase.
        Another aspect of the invention provides a
30 recombinant method of regulating transcription of a gene
of interest. In this aspect, the transcription of a gene
downstream or even upstream in a signal transduction
pathway from the kinase of the invention is regulated by
an expressible gene construct comprising a promoter and a
35 structural gene s quence for the kinase of the invention

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or a protein that affects the activity of the kinase that
is introduced into the cell where transcription is to be
regulated.

Another aspect of the invention relates to new
5 nuclear kinases that are regulated by signal transduction
and that participate in phosphorylation cascades which
regulate transcription and related methods for regulating
transcription. The kinases are: (i) substantially
exclusively intranuclearly localized; (ii) capable of
10 autophosphorylation; (iii) selectively bindable with
antibodies raised against the RING3 portion of GST-RING3;
(iv) of a molecular weight of from about 82.5 to about
92.7 kilodaltons; and (v) include peptide sequences Asp-
Ser-Asn-Pro-Asp-Glu-Ile-Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-
15 Pro-Thr-Thr-Leu (SEQ ID NO: 1) and Ala-Val-His-Glu-Gln-
Leu-Ala-Ala-Leu-Ser-Gln-Ala-Pro (SEQ ID NO: 2). Also
contemplated are biologically active fragment
derivatives, analogs, conjugates, and fusion proteins
thereof.

20   As another aspect of the invention, protein kinase
activity has now been discovered for RING3 (human).
RING3 is attributed with a role consistent with that of
the novel kinases.

Another aspect of the invention relates to the
25 recognition of the relevance of the novel kinases(s) of
the invention and RING3 to (i) the regulation or control
of the cell cycle, (ii) control of cell differentiation,
and (iii) control of cell growth and metabolism.

In another aspect, the invention features gene
30 therapy employing DNA encoding the 90 kD kinase (or
RING3).   Where overexpression or overactivation of the
90 kD kinase is associated with a disease state, an
antisense RNA product encoding all or part of the 90 kD
kinase may be used to reduce expression of the 90 kD
35 kinase. The antisense RNA by be administered, for

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example, by expression from a retroviral vector
delivered, for example, to the bone marrow. Treatment
may be combined with more traditional cancer therapies
such as surgery, radiation, or other forms of
5 chemotherapy. In other disease states it may be
desirable to increase expression of the 90 kD kinase.
For example, in AIDS patients it may be desirable to
increase proliferation of certain classes of lymphocytes
(e.g. CD4+ cells). Introduction of a gene expressing (or
10 overexpressing) active 90 kD kinase into CD4+ cells by
gene therapy may increase proliferation of these cells.
For example, CD4+ cells could be withdrawn from a patient
and treated to remove all traces of HIV. The cells would
then be transfected with a virus encoding 90 kD kinase.
15 The CD4+ cells would then be reintroduced into the
patient.
          In other embodiments, the invention includes
proteins which are substantially identical to a human 90
kD kinase such homologs include other substantially pure
20 naturally occurring mammalian 90 kD kinases as well as
allelic variants; natural mutants; induced mutants;
proteins encoded by DNA that hybridizes to the 90 kD
kinase DNA sequence under high stringency conditions or
low stringency conditions (e.g., washing at 2X SSC at
25 40°C with a probe length of at least 40 nucleotides); and
polypeptides or proteins specifically bound by antisera
directed to a 90 kD kinase. The term also includes
chimeric polypeptides that include a 90 kD kinase
fragment.
30   The invention further includes analogs of any
naturally occurring 90 kD kinase. Analogs can differ
from the naturally occurring 90 kD kinase by amino acid
sequence differences, by post-translational
modifications, or by both. Analogs of the invention will
35 generally exhibit at least 85%, more preferably 90%, and

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most preferably 95% or even 99% identity with all or part
of a naturally occurring 90 kD kinase sequence. The
length of comparison sequences will be at least 15 amino
acid residues, preferably at least 25 amino acid
5   residues, and more preferably more than 35 amino acid
residues. Modifications include *in vivo* and *in vitro*
chemical derivatization of polypeptides, e.g.,
acetylation, carboxylation, phosphorylation, or
glycosylation; such modifications may occur during
10 polypeptide synthesis or processing or following
treatment with isolated modifying enzymes. Analogs can
also differ from the naturally occurring 90 kD kinase by
alterations in primary sequence. These include genetic
variants, both natural and induced (for example,
15 resulting from random mutagenesis by irradiation or
exposure to ethanemethylsulfate or by site-specific
mutagenesis as described Ausubel et al., 1994. Also
included are cyclized peptides molecules and analogs
which contain residues other than L-amino acids, e.g., D-
20 amino acids or non-naturally occurring or synthetic amino
acids, e.g., β or γ amino acids.

     In addition to full-length polypeptides, the
invention also includes 90 kD kinase fragments. As used
herein, the term "fragment," means at least 20 contiguous
25 amino acids, preferably at least 30 contiguous amino
acids, more preferably at least 50 contiguous amino
acids, and most preferably at least 60 to 80 or more
contiguous amino acids. Fragments of 90 kD kinases can
be generated by methods known to those skilled in the art
30 or may result from normal protein processing (e.g.,
removal of amino acids from the nascent polypeptide that
are not required for biological activity or removal of
amino acids by alternative mRNA splicing or alternative
protein processing events).

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In another aspect the invention features a 90 kD
kinase assay for cancers other than leukemias. Levels of
active (autophosphorylated) 90 kD kinase (or RING3) may
be elevated in a wide range of cancers, including:
5   melanomas, hepatocarcinomas, astrocytomas, lymphomas, and
epithelial cancers. For these and other cancers,
diagnostic assays simialr to those outlined above for the
detection of leukemic cells will be useful.

All publications and patent applications mentioned
10 in this specification are herein incorporated by
reference to the same extent as if each independent
publication or patent application was specifically and
individually indicated to be incorporated by reference.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER

    (ii) TITLE OF INVENTION: NUCLEAR LOCALIZED TRANSCRIPTION FACTOR
        KINASES AND DIAGNOSTIC ASSAYS RELATED THERETO

    (iii) NUMBER OF SEQUENCES: 25

    (iv) CORRESPONDENCE ADDRESS:
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        (B) STREET: 225 Franklin Street
        (C) CITY: Boston
        (D) STATE: MA
        (E) COUNTRY: USA
        (F) ZIP: 02110-2804

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: PCT/US96/-----
        (B) FILING DATE: 03-JUL-1996
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/503,062
        (B) FILING DATE: 10-JUL-1995
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
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        (B) REGISTRATION NUMBER: 32,983
        (C) REFERENCE/DOCKET NUMBER: 04020/080WO1

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 617/542-5070
        (B) TELEFAX: 617/542-8906
        (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Asp Ser Asn Pro Asp Glu Ile Glu Ile Asp Phe Glu Thr Leu Lys Pro
1               5                   10                  15

Thr Thr Leu
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

    Ala Val His Glu Gln Leu Ala Ala Leu Ser Gln Ala Pro
    1   5   10

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

    Lys Lys Lys Arg Lys
    1   5

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

    Gly Xaa Xaa Xaa Xaa Gly Lys
    1   5

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    Gly Xaa Gly Xaa Xaa Gly
    1   5

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(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Gly Ala Gly Ser Val Gly Gly Val Gly Gly Ala Gly Ala Ala Gly Gly
1   5   10   15

Gly Asn Ala Ser Lys
          20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Ala Gly Gly Phe Gly Ser Val Tyr Lys Ala Thr Tyr Arg Gly Val
1   5   10   15

Pro Val Ala Ile Lys
          20

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Tyr His Asp Ile Ile Lys Xaa Pro Xaa Xaa Leu
1   5   10

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Pro Glu Phe
1

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gln Glu Phe
1

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Asp Val Val Ala Met Xaa Arg Lys Leu
1   5

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Tyr Ala Lys Met
1

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
His Arg Leu Ala Glu Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa His Glu Gln
1               5                   10                  15
```

```
Leu Ala Ala
```

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Arg Arg Leu Ser Ser Arg Ala
1               5
```

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ala Pro Arg Thr Pro Gly Gly Arg Arg
1               5
```

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Arg Arg Arg Glu Glu Glu Thr Glu Glu Glu
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 13 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Arg Phe Ala Arg Lys Gly Ser Leu Arg Gln Lys Asn Val
1   5   10

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 10 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Pro Leu Ser Arg Thr Leu Ser Val Ser Ser
1   5   10

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 13 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu Leu Arg Pro Gln Arg Ala Thr Ser Asn Val Phe Ser
1   5   10

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 7 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Leu Arg Arg Ala Ser Leu Gly
1   5

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 19 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: not relevant
      (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Asp Ser Asn Pro Asp Glu Ile Glu Ile Asp Phe Glu Thr Leu Lys Pro
1   5   10   15

Thr Thr Leu

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 13 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

        Ala Val His Glu Gln Leu Ala Ala Leu Ser Gln Ala Pro
        1   5   10

(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 6 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

        Gly Xaa Gly Xaa Xaa Gly
        1   5

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGCCGCGGAT CCATGGCTTC GGTGCCTGCT
30

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCTGGGAAT TCAATGTT
18

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What is claimed is:

1.   A method of detecting leukemic cells in a
biological sample, said method comprising measuring the
activity of a RING3-related kinase in said sample, an
5   increase in said activity relative to a control being
indicative of the presence of leukemic cells.

2.   The method of claim 1 wherein said biological
sample comprises lymphocytes.

3.   The method of claim 1 wherein said activity
10   is measured by assaying the autophosphorylation activity
of a RING3-related kinase.

4.   The method of claim 1 wherein said activity
is measured by assaying phosphorylation of a substrate of
a RING3-related kinase.

15   5.   The method of claim 3 wherein
autophosphorylation activity is measured by immunological
methods.

6.   A kit for detecting leukemic cells in a
biological sample, said kit comprising a substantially
20   pure antibody that specifically recognizes an
autophosphorylated form of a RING3-related kinase.

7.   The method of claim 1 wherein said RING3-
related kinase activity is measured in a first and a
second biological sample, a higher level of RING3-related
25   kinase activity in said first biological sample relative
to said second biological sample indicating the presence
of more leukemic cells in said first biological sample
than in said second biological sample.

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8.   The method of claim 7 wherein said first and second biological samples are obtained from the same patient at different times.

9.   The method of claim 7 wherein said second biological sample is obtained from an individual that is not suffering from a leukemic condition.

10.   The method of claim 1 wherein said activity is measured by assaying the phosphorylation activity of a RING3-related kinase.

11.   The method of claim 1 wherein said RING3-related kinase is the 90 kD kinase.

12.   The method of claim 1 wherein said RING3-related kinase is RING3.

] 84.5 - 92.7 kDa

Figure 1A

time (minutes)

0 3 6 9 12 15 30

Figure 1B

control
EGF
serum
forskolin

kDa

— 100.6

— 71.2

— 43.5
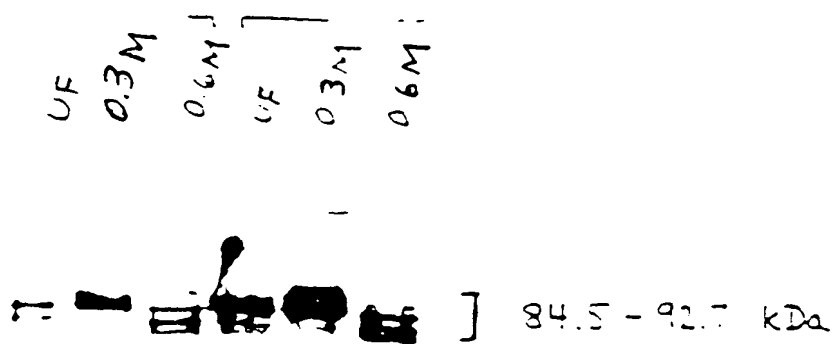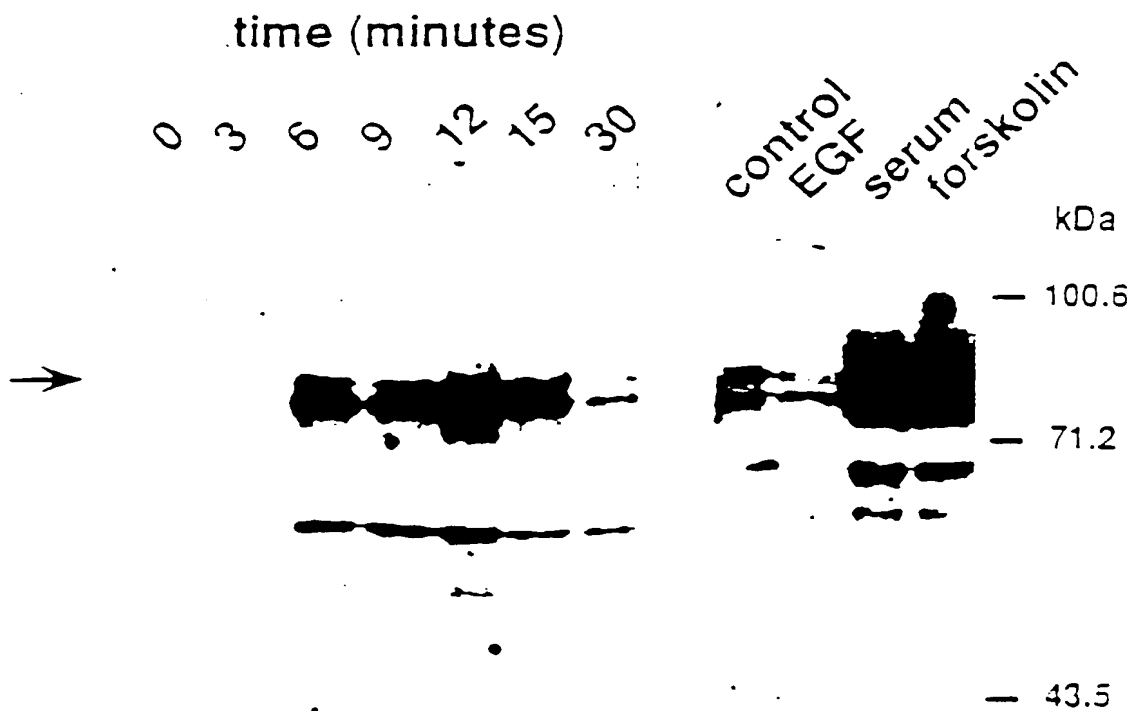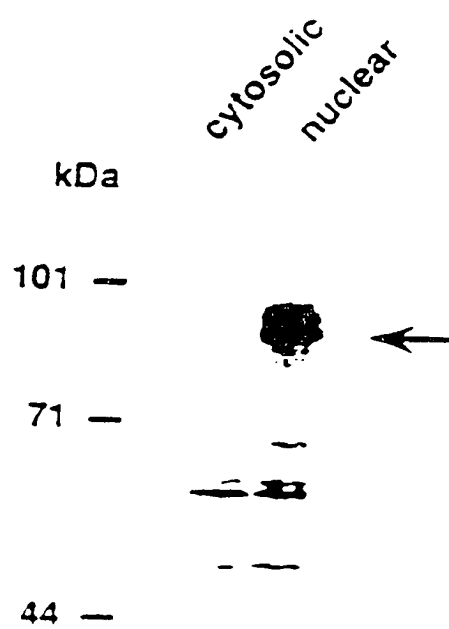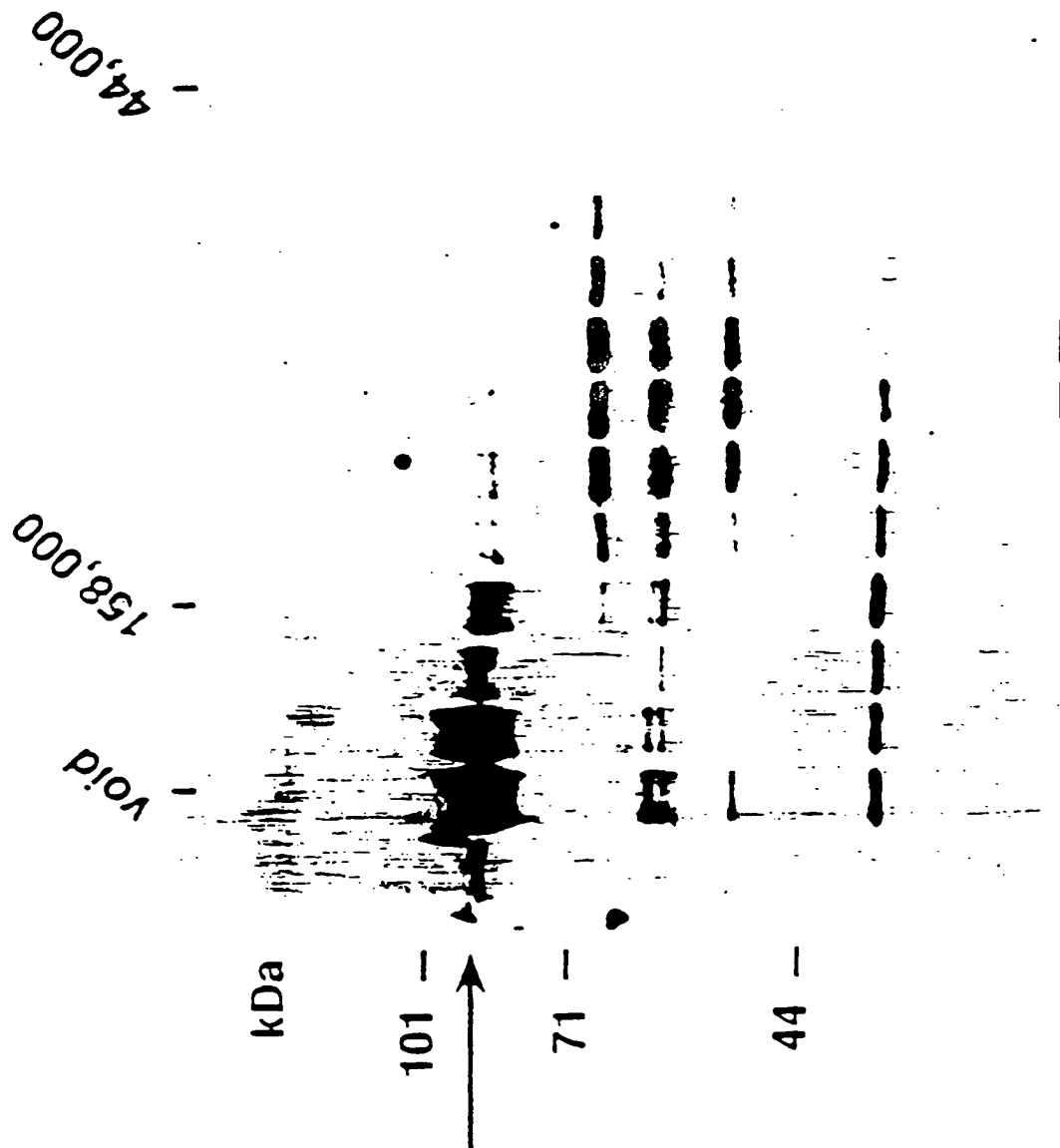
Figure 1C

Figure 2

Figure 3
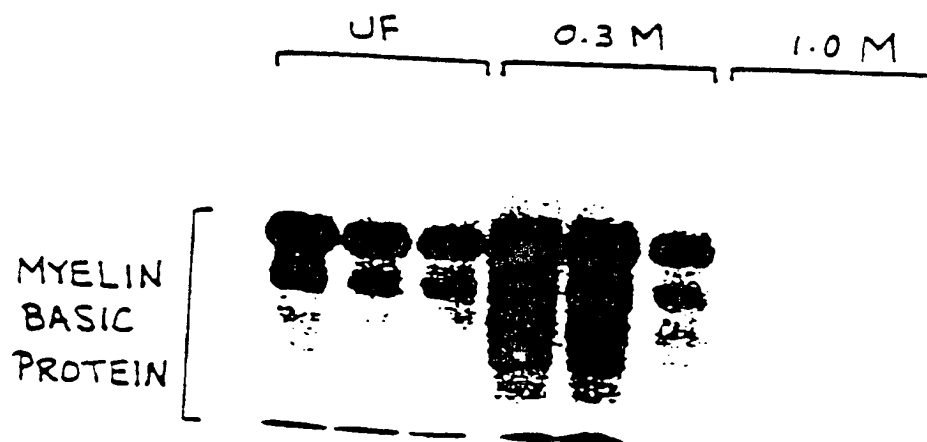
Figure 4
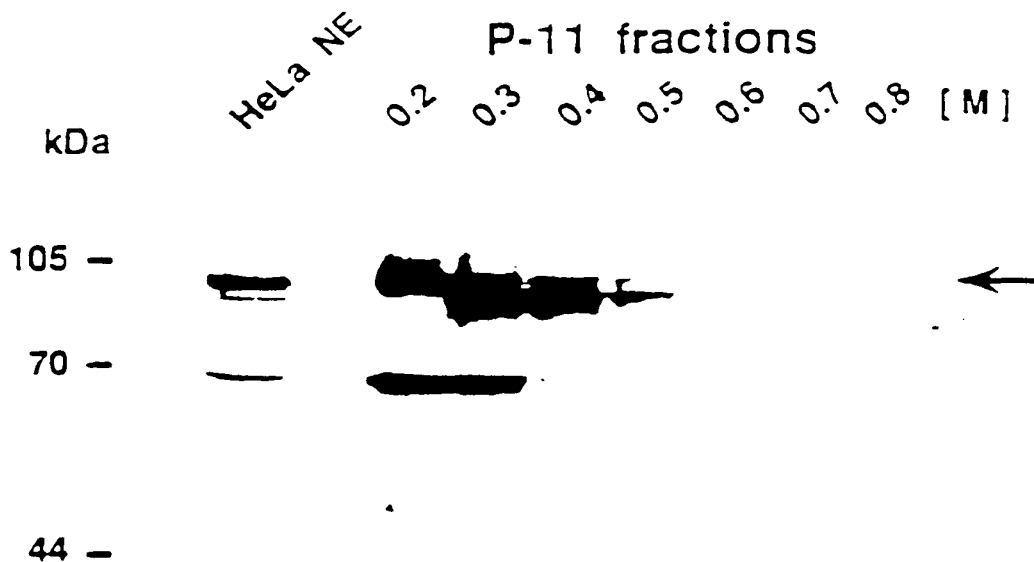
Figure 5A
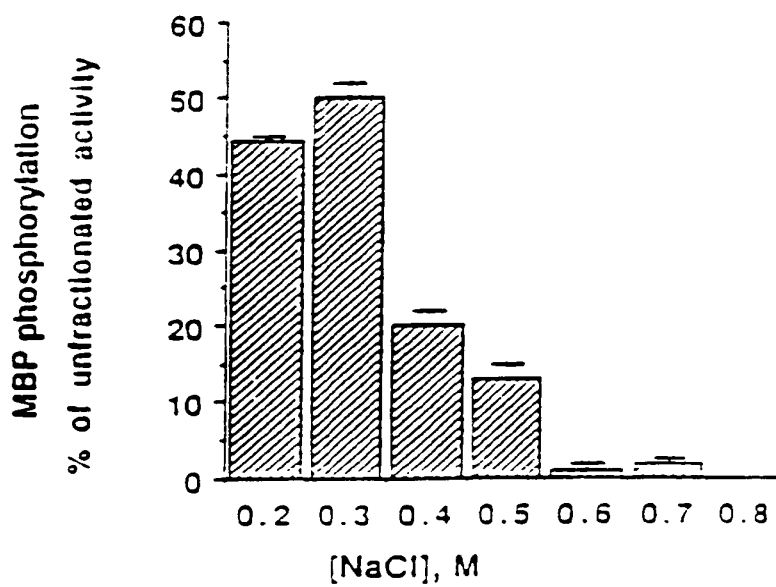
Figure 5B

Figure 6A
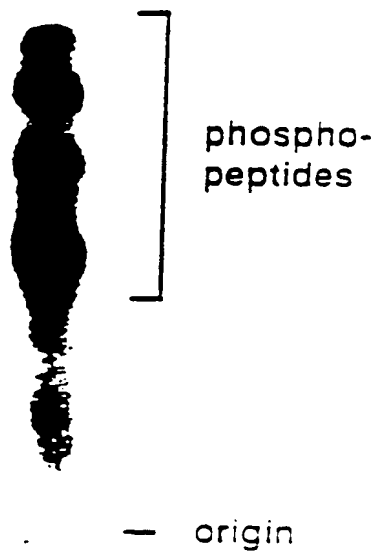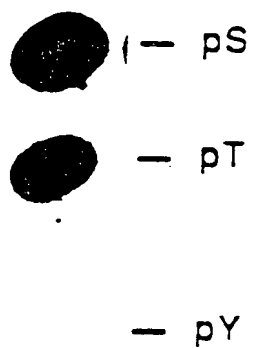
Figure 6B
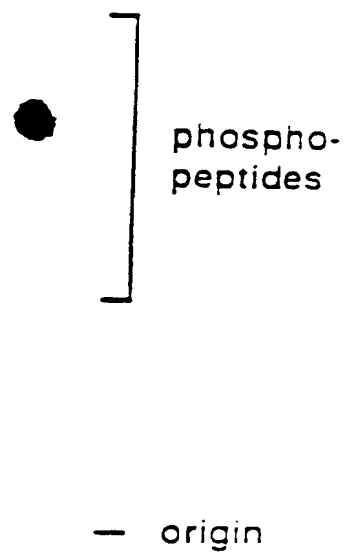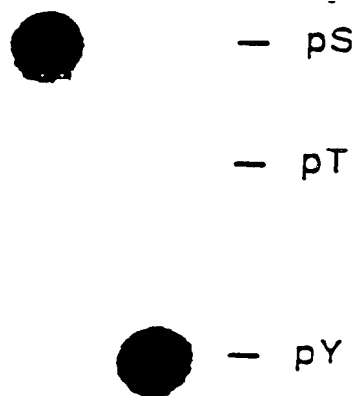
Autophosphorylation
of immune complex
from HeLa NE

preimmune
immune α RING 3

☰ ← 90 kDa kinase

preimmune
immune α fsh

= ← 90 kDa kinase

Figure 7

Figure 8

FIG. 9

Renatured cell extracts

Renatured αRING3
immune complexes

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(57) Abstract

New nuclear kinases that are regulated by signal transduction and that participate in phosphorylation cascades which regulate transcription and related methods for regulating transcription. The novel nuclear kinases play a vital role in gene expression, particularly with regard to leukemia in humans. The kinase is (i) substantially exclusively intranuclearly localized; (ii) capable of autophosphorylation; (iii) selectively bindable with antibodies raised against the RING3 portion of GST-RING3; (iv) of a molecular weight of from about 82.5 to about 92.7 kilodaltons; and (v) includes peptide sequences Asp-Ser-Asn Pro-Asp-Glu-Ile-Asp-Phe-Glu-Thr-Leu-Lys-Pro-Thr-Thr-Leu and Ala-Val-His-Glu-Gln-Leu-Ala-Ala-Leu-Ser-Gln-Ala-Pro. The invention features a method of detecting leukemic cells in a biological sample, the method includes measuring the activity of a RING3-related kinase in the sample, an increase in the activity relative to a control being indicative of the presence of leukemic cells.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/00; G01N 33/53
US CL :435/4, 7.1, 7.3; 436/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 7.1, 7.3; 436/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, Biosis, CAPlus, WPIDS, JAPIO, PATOSEP, PATOSWO
search terms: RING3, kinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	DENIS et al. A novel, mitogen-activated nuclear kinase is related to a Drosophila developmental regulator. Genes Development, December 1995. Vol. 10. pages 261-271.	1-12

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search	Date of mailing of the international search report
13 JANUARY 1997	3 1 JAN 1997

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